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REVIEW

Changing trends in biotechnology of secondary metabolism in medicinal and aromatic plants

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Abstract

Main conclusion **Medicinal and aromatic plants are known to produce secondary metabolites that find uses as flavoring agents, fragrances, insecticides, dyes and drugs. Biotechnology offers several choices through which secondary metabolism in medicinal plants can be altered in innovative ways, to overproduce phytochemicals of interest, to reduce the content of toxic compounds or even to produce novel chemicals. Detailed investigation of chromatin organization and microRNAs affecting biosynthesis of secondary metabolites as well as exploring cryptic biosynthetic clusters and synthetic biology options, may provide additional ways to harness this resource.**

Plant secondary metabolites are a fascinating class of phytochemicals exhibiting immense chemical diversity. Considerable enigma regarding their natural biological functions and the vast array of pharmacological activities, amongst other uses, make secondary metabolites interesting and important candidates for research. Here, we present an update on changing trends in the biotechnological approaches that are used to understand and exploit the secondary metabolism in medicinal and aromatic plants. Bioprocessing in the form of suspension culture, organ culture or transformed hairy roots has been successful in scaling up secondary metabolite production in many cases. Pathway elucidation and metabolic engineering have been useful to get enhanced yield of the metabolite of interest; or, for producing novel metabolites. Heterologous expression of putative plant secondary metabolite biosynthesis genes in a microbe is useful to validate their functions, and in some cases, also, to produce plant metabolites in microbes. Endophytes, the microbes that normally colonize plant tissues, may also produce the phytochemicals produced by the host plant. The review also provides perspectives on future research in the field.

Keywords Bioprocessing · Endophyte · Hairy roots · Heterologous expression · Metabolic engineering · Suspension culture

Abbreviations

4CL	4-Coumaroyl:CoA ligase
CHI	Chalcone isomerase
CUS	Curcuminoid synthase
CYPs	Cytochrome P450 enzymes
DMAPP	Dimethylallyl pyrophosphate
EST	Expressed sequenced tag
IPP	Isopentenyl pyrophosphate
mA	Milli ampere (electric current)
MAP	Medicinal and aromatic plants
MEP	Methylerythritolphosphate pathway
MEV	Mevalonate pathway
ORF	Open reading frame
PAL	Phenylalanine ammonia-lyase
PLR	Pinoresinol/lariciresinol reductase
STS	Stilbene synthase

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T DNA	Transfer deoxyribose nucleic acid
TIA	Terpenoid indole alkaloid pathway
US-FDA	United States Food and Drug Administration
UV	Ultra violet
VIGS	Virus induced gene silencing

Introduction

A variety of organic compounds are synthesized by plants, which are chiefly classified as primary and secondary metabolites. Primary metabolites are required for basic processes like photosynthesis, respiration, growth and development. Secondary metabolites are other phytochemicals, which are specifically accumulated and are not present merely as intermediates of chemical processes. These compounds are very diverse and distribution of specific types of secondary metabolites is often restricted to taxonomically related species. Though precise functions of secondary metabolites in plant metabolism and physiology are as yet unclear, they are believed to play various roles in interactions of plants with their environment, like (a) providing protection to plants against pathogens (Schwekendiek et al. 2007; Naoumkina et al. 2008) (b) providing protection against abiotic stresses like UV radiation (Xu et al. 2008) (c) attractants for pollinators (Kessler and Baldwin 2007; González-Teuber and Heil 2009) (d) signal molecules (Xu et al. 2009) etc.

The major reason for interest in plant secondary metabolites stems from their overwhelming diversity. They appear to be a never-ending source of novel chemical structures with a variety of pharmacological activities. Nearly 100,000 such metabolites have been isolated from higher plants (Verpoorte et al. 1999; Afendi et al. 2012). Several of these chemicals are used as flavoring agents, fragrances, insecticides, dyes and drugs. Since time immemorial plants and their products have also been used as traditional medicines for treatment of common ailments (Crozier et al. 2006); an estimate suggests that up to 70,000 species of plants are used in folk medicine (Farnsworth and Soejartto 1991). In India about 7,500 plant species are used in ethnomedicines (Shankar and Majumdar 1997). In China, about 1,000 medicinal plants are commonly used as traditional medicine (He and Sheng 1997). Advances in chemistry and pharmacology have validated or vitiated the claims of traditional medicines and have discovered the active principles. About 50 % of all US-FDA approved drugs introduced in the market are natural products or their analogues (Vuorelaa et al. 2004). However, often the raw material could be limiting and its exploitation may be surrounded by ecological concerns. One of the key objectives of plant biotechnology is to develop eco-friendly

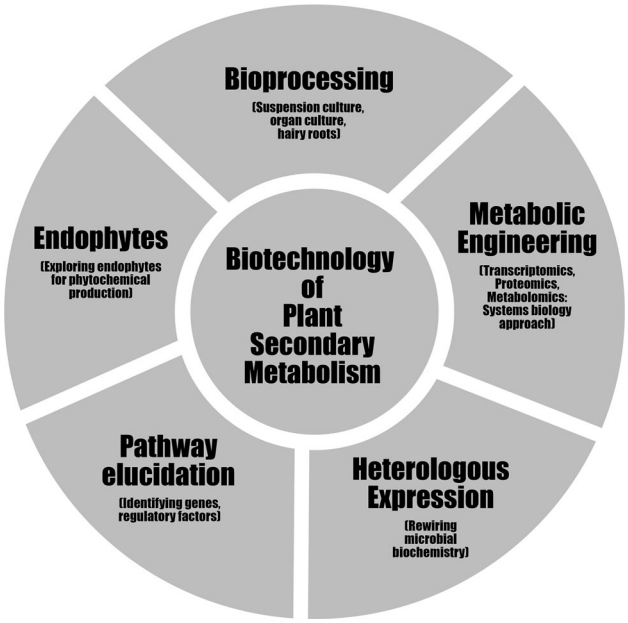

Fig. 1 Biotechnology of plant secondary metabolism

ways of large-scale production of pharmacologically active compounds. Moreover, the enormous biosynthetic potential of plants is yet to be exploited completely and biotechnology could be used to generate novel chemical compounds, with enhanced or newer bioactivities, through activation of silent or cryptic metabolic clusters.

Powerful molecular tools have been used to exploit microbial biochemistry to produce novel compounds (Prather and Martin 2008). This in part, could be attributed to lesser complexity, clustering of genes involved in a pathway, lower redundancy, easy amenability to genetic intervention and availability of genome sequences of a large number of microbes. Biotechnological interventions have also played a major role in improvement of crop yields and quality. Crops have also been engineered to produce valuable enzymes, heterologous proteins and antibodies (Desai et al. 2010). Despite such progress in plant molecular biology, only limited application of biotechnology has been seen in medicinal and aromatic plants (MAPs). In MAPs, generally there is paucity of available molecular information and standardized protocols for transgenesis and marker-assisted selection are also not readily available. In contrast, for most crop species, presence of large EST libraries, genome sequences for at least some of the crops and standardized protocols for transgenesis have played a role in employing biotechnology for improvement of crop yields. However, for MAPs, use of hairy root cultures and bioreactors for production of secondary metabolites have become popular (Srivastava and Srivastava 2007). Moreover, reducing time and costs of de novo genome and EST sequencing have made it possible to

unravel the molecular secrets of secondary metabolite production by MAPs. Here we present an update of biotechnological applications in plant secondary metabolism (Fig. 1). Supplementary Table 1 summarizes the available biotechnologies for few pharmacologically important plant secondary metabolites obtained from MAPs.

Bioprocessing

Plant secondary metabolites are usually produced in lesser quantities; often they get accumulated in specific plant organs, at distinct developmental stages, or, on an exposure to a specific stress, or in a particular agro-geo-climatic zone (Chemler and Koffas 2008). Many such metabolites like taxol, artemisinin, forskolin etc. are very difficult to synthesize chemically, and the process is economically unviable (Hashimoto et al. 1988; Corey et al. 1988; Heinstein and Chang 1994; Bouwmeester et al. 2006). Industrial scale plant tissue culture presents itself as a commercially viable alternative for production of phytochemicals, considering the (1) increasing demand for metabolites of interest, (2) long-time scales required for certain slow growing plants, (3) continuously reducing land availability for large-scale cultivation of plants, and (4) destruction of wild populations of medicinal plants through blatant exploitation.

Suspension culture

Suspension cultures are fast growing and amenable to continuous culture in a chemostat. For establishing plant cell suspension cultures, the undifferentiated plant cell culture or callus is generally transferred into liquid medium and agitated on a rotary shaker. However, plant cell cultures growing in such environments show propensity towards production of certain compounds only (Smetanska 2008). Few biosynthetic pathways, such as those involved in production of cinnamic acid derivatives, anthraquinones, berberines, shikonins, anthocynanins etc., express very well in suspension cultures (Chattopadhyay et al. 2002; Chiang and Abdullah 2007). Many times these compounds get spontaneously accumulated in suspension cell cultures, often at concentrations much higher than found in intact plants, even without any efforts for medium engineering. In contrast, other compounds such as morphinan alkaloids, tropane alkaloids (e.g. hyoscyamine and scopolamine), quinoline alkaloids, dimeric monoterpene indole alkaloids (e.g. vinblastine and vincristine) etc. are expressed only in traces in suspension cultures (Berlin 1997). Large-scale efforts to increase their expression through medium engineering and use of elicitors have not yielded results that can lead to commercial exploitation of tissue cultures for production of these compounds.

Organ culture

Organ culture has been explored for production of such phytochemicals that are not expressed by suspension culture. Morphinan alkaloids of *Papaver somniferum* L. (Papaveraceae), dimeric indole alkaloid (anhydrovinblastine—a direct precursor of vinblastine and vincristine) of *Catharanthus roseus* (L.) G. Don (Apocynaceae), sesquiterpene lactone (artemisinin) of *Artemisia annua* L. (Asteraceae), for instance, are produced in better quantities in shoot culture (Endo et al. 1987; Liu et al. 2003; Tisserat and Berhow 2009). Similarly root cultures produce better amounts of tropane alkaloids, such as hyoscyamine and scopolamine, as compared to suspension cultures (Berlin 1997; Saito and Mizukami 2002).

Hairy root culture

Genetic transformation of plant cells using *Agrobacterium rhizogenes* results in differentiation into hairy roots. These roots can be excised from the infection site, bacteria can be removed using antibiotic treatment, and then the hairy roots can be cultured indefinitely in liquid medium. Hairy roots cultures have several advantages, such as (1) high growth rate, (2) genotypic and phenotypic stability over long culture periods, (3) they do not require exogenous supply of plant growth regulators, and (4) produce high levels of secondary metabolites (Srivastava and Srivastava 2007). Expression levels of genes contained in the inserted T-DNA have been correlated to the amount of secondary metabolites produced. The variability in different insertion lines can be used to select for the lines that are better producers. Apart from phytochemicals, hairy roots have also been explored, though with limited success, for the production of heterologous proteins (Tzfira and Citovsky 2008).

Process optimization

Like any other plant tissue culture process, the use of suspension cells and organ cultures, requires addition of auxin and cytokinins in specific ratio, to either promote dedifferentiation into suspension cells, or in a different ratio for differentiation into specific plant organs. However, if the products (phytochemicals) are to be used as crude extract for human/animal consumption, it is desirable to avoid addition of plant growth regulators. Media optimization also plays a crucial role in production of secondary metabolites. For certain secondary metabolites, a media that supports maximum biomass production, may not actually result in corresponding higher yields of secondary metabolites. For instance, addition of nitrogen-rich medium resulted in 25–30 % increase in biomass but only

marginal (2–9 %) increase in shikonin concentration by *Lithospermum erythrorhizon* Siebold & Zucc. (Boraginaceae) culture (Srinivasan and Ryu 1993). The nature of carbon source used could also affect biomass as well as production of secondary metabolites. Glucose and/or sucrose are considered good carbon sources for biomass increase in plant cell culture, but not for hairy root culture (Giri and Narasu 2000). Carbon–nitrogen ratio also affects secondary metabolite biosynthesis. Nitrogen is usually supplied as ammonia and nitrates. Ratios of these two, as well as total nitrogen content in the medium could affect secondary metabolite production differently in different plant cell cultures. Alternating carbon- and nitrogen-rich media enhanced shikonin production in cultures of *L. erythrorhizon*, while betacyanin production was markedly increased in cultures of *Phytolacca americana* L. (Phytolaccaceae) with increase in total nitrogen supply (Sakuta et al. 1987; Srinivasan and Ryu 1993). Similarly phosphate limitation also reduced anthraquinone synthesis by cultures of *Galium mollugo* L. (Rubiaceae) (Wilson and Balague 1985). Production of secondary metabolites by plant cell cultures is also greatly potentiated by addition of biotic (of biological origin) or abiotic (chemical or physical) elicitors (Karuppusamy 2010). Use of abiotic elicitors is thought to induce production of phytoallexins and release of biotic elicitors from plant cell walls (Davis et al. 1986). Sequential treatment of commonly used elicitors such as methyl jasmonate (abiotic), salicylic acid (abiotic) and yeast extract (biotic) at 24 h intervals was found to enhance the accumulation of dihydrosanguinarine (2.5 times) and sanguinarine (5.5 times) in *Eschscholzia californica* Cham. (Papaveraceae) suspension culture (Cho et al. 2008). Addition of sodium vanadate and vanadyl sulfate was found to increase the production of coumarins in the suspension culture of *Angelica archangelica* L. (Apiaceae) (Siatka and Kasparová 2007). Use of UV-B light leads to increase in production of catharanthine in *Catharanthus roseus* cell suspension culture and flavonoid production in *Passiflora quadrangularis* L. (Passifloraceae) callus culture (Ramani and Chelliah 2007; Antognoni et al. 2007). Addition of filtered and autoclaved mycelial extract of *Verticillium dahliae* increased the production of artemisinin from hairy root cultures of *A. annua* without affecting the growth and morphology of hairy roots (Wang et al. 2000a, b). Electric current also appears to be a good elicitor for secondary metabolite production. Pea hairy roots treated with 30–100 mA of electric current produced 13 times higher amounts of (+)-pisatin compared to the non-elicited controls. Similarly seedlings, intact roots or cell suspension cultures of *Trigonella foenum-graecum* L. (Fabaceae), *Medicago truncatula* Gaertn. (Fabaceae), *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae), *Trifolium pratense* L. (Fabaceae) and *Cicer arietinum* L. (Fabaceae) also produced increased levels of secondary metabolites in response to electro-elicitation (Kaimoyo et al. 2008).

Several types of bioreactors have been used for production of secondary metabolites by plant cell cultures. The general aspects that need to be taken care of during bioreactor design are (1) low shear mixing for efficient nutrient transport without sedimentation or clumping of cells, (2) optimal aeration with low shear stress, (3) sterility of the process, and (4) introduction of light for phototrophic cultures. Stirred reactor, rotating drum reactor, fluidized bed reactor, airlift reactor, etc. have been used for both suspension cells and hairy root cultures. Bubble column and aerated reactors were found to be more suitable for organ cultures. Souret et al. (2003) compared terpenoid gene expression patterns and artemisinin production from hairy root cultures of *A. annua* in shake flask, bubble column reactor and mist reactor. They found that bubble column reactor supported more biomass production while more artemisinin was produced in mist reactor. Moreover, root samples from different regions of the same reactor showed considerable differences in expression of terpenoid pathway genes. Scale-up to bioreactors as well as the choice of bioreactor continues to remain a challenge. Mathematical models have been used to evaluate the process parameters against productivity and provide optimal conditions using different types of bioreactors (Rizvi 2012).

Molecular elucidation of plant secondary metabolite pathways

Molecular elucidation in broad sense consists of finding out the precise chemical route of metabolite biosynthesis, enzymes catalyzing the biosynthetic reactions, genes encoding the biosynthetic enzymes and regulatory factors that control secondary metabolite biosynthesis. Identification of genes involved in plant secondary metabolite biosynthesis is a very important component of biotechnology of MAPs. The availability of molecular information with regards to production and regulation of plant secondary metabolites enables the biotechnologist to rationally tinker with the biosynthetic machinery. Approaches used to study secondary metabolite pathways have changed considerably over time, with the availability of new molecular tools and technological advances. These approaches may be roughly divided into pre-genomic era and post-genomic era approaches.

Precursor labeling

Labeling experiments and retro biosynthetic studies generally precede the identification of enzymes and genes

involved in secondary metabolite biosynthesis. These are used to trace the precise chemical route of biosynthesis. For instance, terpenoids that contribute one-third of all known secondary metabolites were shown to be produced by condensation of C5 units—IPP (isopentenyl pyrophosphate) and DMAPP (dimethylallyl pyrophosphate) (Poulter et al. 1981). Earlier it was thought that only the cytosolic mevalonate (MEV) pathway produces IPP, the universal precursor of all terpenoids. However, with the use of ^{13}C labeled intermediates, it was shown that in microorganisms and plants, certain terpenoids are produced not from mevalonate pathways, but from another pathway also producing IPP/DMAPP (Rohmer 1999). Now it is well established that cytosolic mevalonate pathway provides precursors for synthesis of sesquiterpenes (C15) and triterpenes (C30) while plastidial methylerythritol phosphate (MEP) pathway provides precursors for synthesis of monoterpenes (C10), diterpenes (C20) and tetraterpenes (C40) (Dudareva et al. 2005).

Pre-genomic era approaches

Biochemical approach

Biochemical approach for molecular dissection of secondary metabolite pathways has been very useful in the pre-genomic era. Here, once the chemical route of metabolite biosynthesis is known, a hypothetical scheme is laid, based on the plausible reaction mechanisms. Enzyme activity is detected in cell free systems and then one proceeds for activity-guided purification of the enzyme using various chromatographic techniques. The purified protein (enzyme) is sequenced, degenerate primers are designed and partial cDNA is amplified using polymerase chain reaction (PCR). The sequence of partial cDNA is used to design RACE (Rapid amplification of cDNA ends) primers and full-length cDNA is cloned. Heterologously expressed protein is checked for the enzyme activity against purified substrates. Phenylalanine aminomutase that catalyzes the first committed step in taxol side-chain biosynthesis was cloned from *Taxus chinensis* Roxb. (Taxaceae) using this approach (Steele et al. 2005). Similarly, phenylalanine ammonia lyase that catalyzes the first committed step in phenylpropanoid biosynthesis was cloned from *Pinus taeda* L. (Pinaceae) using this method (Whetten and Sederoff 1992).

Positional cloning, tagging and expression libraries

Another pre-genomic era approach, involves positional cloning of biosynthetic pathway genes. This approach starts with creation of mutants that are defective in secondary metabolite synthesis, mainly those metabolites whose deficiency results in score able phenotypes, such as color, aroma and flavor. Mutants are classified into complementation groups and map-based cloning of gene ensues. The open reading frames in the cloned DNA are expressed in heterologous system and assayed for enzyme activity. To cite an illustration, these methods were used in discovery of an alternative pathway for formation of β-carotene in plant chloroplasts. Two mutations that affect tomato pigmentation: Beta, a dominant mutation that increases β-carotene and old gold, a recessive mutation that stops β-carotene synthesis and increases lycopene production, were analyzed. Positional cloning and further molecular analysis revealed that Beta encoded a lycopene β-cyclase that converts lycopene to carotene. Old gold was found to be a null allele of Beta (Ronen et al. 2000).

Alternatively, a functionally expressed cDNA library is screened for the requisite enzyme activity against purified substrates. Once the expected enzyme activity is detected, the clone is sequenced. This approach has been used for cloning of several cytochrome P450 enzymes, that catalyze various steps in many secondary metabolite pathways (Schoendorf et al. 2001).

Homology based cloning

Once large number of sequences were accumulated using the above-mentioned methods, it became evident, that related enzymes share considerable sequence homology both at protein and DNA level, at least in conserved domains, which could be used for designing degenerate primers and cloning of related genes in new plant species. This approach considerably reduced the time required for cloning of secondary metabolite pathway genes, and has been successfully employed for several important plant secondary metabolites. To exemplify, β-caryophyllene synthase of *A. annua* which converts farnesyl diphosphate to β-caryophyllene, was cloned using this approach (Cai et al. 2002). Similarly, Engprasert et al. (2004) aligned the protein sequences of geranylgeranyl diphosphate synthase and identified regions of high homology. Degenerate primers were used to amplify partial GGPP synthase gene and eventually full-length cDNA was cloned from *Coleus forskohlii* (Willd.) Briq. (Lamiaceae).

Post-genomic era approaches

Differential expression analysis, EST libraries, NGS

In the post-genomics era, the reducing costs of DNA sequencing, availability of large-scale proteomics platforms and development of better bioinformatics tools have changed the outlook and approaches to understand the plant secondary metabolite pathways at a molecular level.

Often the secondary metabolites are synthesized in specific plant organs, for instance, leaf trichomes are sites of synthesis of several secondary metabolites (Lommen et al. 2006). This property is exploited for conducting a differential expression-based transcriptomics study. Treatment with biotic or abiotic elicitors that induce production of specific secondary metabolites could also present an opportunity for conduction of a differential expression study. Suppression subtractive hybridization is one such method, following which the EST library is sequenced. Recently the advances in massively parallel sequencing techniques (next generation sequencing platforms like Roche 454®, Illumina Solexa® etc.) have considerably reduced the time required for sequencing of differentially expressed transcriptomes. Wherever genomic resources preexist, a microarray-based differential expression study may be conducted. Differentially expressed RNAs (or proteins, in case of comparison of 2D PAGE profiles) are analyzed by bioinformatic tools. Genes, which could be involved in the biosynthesis of secondary metabolite in question, are identified on the basis of homology. Further their expression pattern helps to predict with some degree of certainty, whether they could be involved in secondary metabolite biosynthesis. Once the genes are predicted, one generally goes for fishing out the full-length cDNA and heterologous expression followed by in vitro enzyme activity determination. To further prove the role of the gene-of-interest in secondary metabolite biosynthesis, knock-out or knock-down lines may be created using transgenesis and then accumulation of preceding intermediate may be tested, as per the proposed biosynthetic pathway. These methods have been employed for characterizing several secondary metabolite pathway genes such as those involved in the production of anti-cancer compounds—vincristine and vinblastine in *C. roseus* (Rischer et al. 2006; Miettinen et al. 2014). Co-expression of secondary metabolite or essential oil components with specific ESTs have also been used for associating functions of genes with metabolites (Fang et al. 2014; Mahajan et al. 2015). Transcriptome sequencing on Roche 454® platform was recently used to better understand the regulation of artemisinin (anti-malarial) metabolism in *A. annua* (Soetaert et al. 2013).

Functional genomics

Reverse genetics has become a popular tool for functional genomics and could be utilized for molecular elucidation of secondary metabolite pathways. Once an EST library or a plant genome is sequenced (and ORFs predicted), genome-scale approaches, mostly utilizing the power of RNA interference-based knock-down, can be employed to find the function of genes (Alonso and Ecker 2006). However, many times the secondary metabolite of interest is produced by an exotic plant species or in some cases by trees, where these methods may not be viable due to unavailability of transgenesis protocols and unreasonable time scales.

Metabolic engineering of plant secondary metabolism

A thorough understanding of biosynthetic machinery and regulatory aspects of plant secondary metabolism are critical for rational metabolic engineering. The biosynthetic processes in a cell are highly networked and several possible fates are possible with tens or hundreds of interactions at each step of a biosynthetic pathway often leading to unpredictable results in metabolic engineering. A systems biology approach, integrating information from metabolomics, proteomics and transcriptomics enables the biotechnologist to engineer the metabolic pathways with higher chances of predictable results (Yang et al. 2014). The main objectives of metabolic engineering of secondary metabolite pathways are to produce novel metabolites, to over produce selective metabolites, to reduce the percentage of toxic and unwanted chemicals and to engineer the biosynthetic apparatus into a microorganism for cheaper, large-scale production of plant secondary metabolites (refer Table 1 for examples).

Overexpression of the enzyme catalyzing the rate-limiting step in a pathway is often used as a strategy to increase the metabolic flux through a pathway. For instance, overexpression of strictosidine synthase, an early enzyme in alkaloid biosynthetic pathway, in *C. roseus* cells leads to increased accumulation of alkaloids (Whitmer et al. 1998). Scopolamine, a medicinally important compound produced by several solanaceous species, is produced by the oxidation of hyoscyamine to scopolamine. Hyocyamine 6β-hydroxylase catalyzes the oxidative reactions that lead to conversion of hyocyamine to scopolamine (Hashimoto et al. 1987). Simultaneous introduction and overexpression of *hyocyamine 6β-hydroxylase* and *putrescine N-methyltransferase* in transgenic *Hyoscyamus niger* L. (Solanaceae) hairy root line resulted in almost nine times higher yields of scopolamine as compared to wild type (Zhang et al. 2004). Similarly, higher levels of anthocyanins and flavonoids are desirable in food products, since these have antioxidant activity. Chalcone isomerase (CHI) is an early enzyme of flavonoid biosynthesis. Overexpression of *CHI* (cloned from *Petunia*) in tomato plants led to a 78-fold increase of flavonoid levels compared to control (Muir et al. 2001). Overexpression of *farnesyl diphosphate synthase* in *A. annua* led to 2–3 fold increase in artemisinin production (Chen et al. 2000). Another approach for increasing metabolic flux is to inhibit competitive pathways. Blocking a

Table 1 Metabolic engineering of plant secondary metabolism

Name of compound	Source plant	Approach used	Gene(s) involved	Results	References
Alkaloids	*Catharanthus roseus* (L.) G.Don	Overexpression of pathway gene	*Strictosidine synthase*	Increased content	Whitmer et al. (1998)
Scopolamine	*Hyoscyamus niger* L.	Overexpression of pathway gene	*Hyocyamine 6b-hydroxylase* and *putrescine N-methyltransferase*	Increased content	Zhang et al. (2004)
Flavonoids	*Solanum lycopersicum* L.	Overexpression of pathway gene	*Chalcone isomerase*	Increased content	Muir et al. (2001)
Artemisinin	*Artemisia annua* L.	Overexpression of pathway gene	*Farnesyl diphosphate synthase*	Increased content	Chen et al. (2000)
Menthol	*Mentha* × *piperita* L.	Inhibiting competitive pathway (through gene silencing/RNAi)	*Menthofuran synthase*	Increased content	Mahmoud and Croteau (2001)
Pinoresinol	*Forsythia koreana* (Rehder) Nakai	Inhibiting competitive pathway (through gene silencing/RNAi)	*Pinoresinol/lariciresinol reductase* (PLR)	Increased content	Kim et al. (2009)
Reticuline	*Papaver somniferum* L.	Inhibiting competitive pathway (through gene silencing/RNAi)	*Codeinone reductase*	Increased content	Allen et al. (2004)
Apocarotenoids and flavonoids	*Solanum lycopersicum* L.	Engineering regulatory mechanism (through gene silencing/RNAi)	*De-etiolated1* (*DET1*)	Increased content	Davuluri et al. (2005)
Flavonoids	*Zea mays* L.	Engineering regulatory mechanism (through overexpression)	*Leaf color* (Lc)	Increased content	Li et al. (2007)
Anthocyanins	*Zea mays* L.	Engineering regulatory mechanism (through overexpression)	C1 and R (transcription factors)	Increased content	Grotewold et al. (1998)
Anthocyanins	*Arabidopsis thaliana* (L.) Heynh., *Nicotiana tabacum* L.	Engineering regulatory mechanism (through overexpression)	C1 and R (transcription factors)	Increased content	Lloyd et al. (1992)
Anthocyanins	*Solanum tuberosum* L.	Engineering regulatory mechanism (through overexpression)	WD40-repeat gene (*StAN11*)	Increased content	Li et al. (2014)
Anthocyanins	*Fragaria* × *ananassa*, cv. Sachinoka	Engineering regulatory mechanism (through overexpression)	FaPHOT2 (phototropin)	Increased content	Kadomura-Ishikawa et al. (2013)
		Engineering regulatory mechanism (through gene silencing/RNAi)		Decreased content	
Alkaloids	*Catharanthus roseus* (L.) G.Don	Engineering regulatory mechanism (through overexpression)	Octadecanoid-derivativeResponsive C atharanthus AP2-domain protein (ORCA3) and geraniol 10-hydroxylase (G10H)	Increased content	van der Fits and Memelink (2000)
Nornicotine	*Nicotiana tabacum* L.	Gene silencing (RNA interference)	CYP82E4	Decreased content	Gavilano et al. (2006)
			Nicotine demethylase	Decreased content	Lewis et al. (2008)
Heavy metals (Lead, cadmium)	*Arabidopsis thaliana* (L.) Heynh.	Heterologous expression	*Pb*(II)/*Cd*(II)/*Zn*(II)-*transporting ATPase* (ZntA)	Improved heavy metal resistance	Lee et al. (2003)
Caffeine	*Coffea arabica L.*	Gene silencing (RNA interference)	*Theobromine synthase* (*CaXMT1*, *CaMXMT1*) and caffeine synthase (*CaDXMT1*)	Decreased caffeine content	Ogita et al. (2003)

Table 1 continued

Name of compound	Source plant	Approach used	Gene(s) involved	Results	References
p-Hydroxybenzylglucosinolates	*Arabidopsis thaliana* (L.) Heynh.	Heterologous expression	CYP79A1	Production of novel compound	Bak et al. (1999)
Laudanine	*Eschscholzia californica* Cham.	Gene silencing (RNA interference)	*Berberine bridge forming enzyme* (BBE)	Production of novel compound	Fujii et al. (2007)
Valencene	*Nicotiana benthamiana* Domin	Inhibiting competitive pathway (through gene silencing/RNAi)	*5-Epi-aristolochene synthase* (*EAS*) and *squalene synthase*	Increased content	Cankar et al. (2014)
Morphine	*Papaver bracteatum* Lindl.	Overexpression of pathway gene	*Codeinone reductase*	Increased content	Sharafi et al. (2013)
Rosmarinic acid	*Salvia miltiorrhiza* Bunge	Engineering regulatory mechanism (through overexpression)	R2R3 MYB (transcriptional repressor)	Decreased content	Zhang et al. (2013)
		Engineering regulatory mechanism (through gene silencing/RNAi)		Increased content	
		Gene silencing (RNA interference)	*Phenylalanine ammonia-lyase* (*PAL*)	Decreased content	Song and Wang (2011)
Apocarotenoids	*Medicago truncatula* Gaertn.	Gene silencing (RNA interference)	*1-Deoxy*-D-*xylulose 5-phosphate synthase 2*	Decreased content	Floss et al. (2008)
Lignin	*Populus tomentosa* Carr.	Engineering regulatory mechanism (through overexpression)	PtoMYB216 (R2R3-MYB transcription factor)	Ectopic deposition of lignin	Tian et al. (2013)
Quercetin 3,4′-diglucoside	*Scutellaria baicalensis* Georgi	Engineering regulatory mechanism (through overexpression)	(SbMYB2 or SbMYB7) R2R3-MYB transcription factors	Decreased content	Yuan et al. (2013)
Tanshinone	*Salvia miltiorrhiza* Bunge	Overexpression of pathway gene	*Allene oxide cyclase*	Increased content	Gu et al. (2012)
Camptothecin	*Camptotheca acuminata* Decne.	Engineering regulatory mechanism (through overexpression)	*Allene oxide cyclase*	Increased content	Yan et al. (2012)
Anthraquinones	*Rubia cordifolia* L.	Engineering regulatory mechanism (through overexpression)	*AtCPK1* (*Arabidopsis* Calcium-dependent kinase gene)	Increased content	Shkryl et al. (2011)

competitive branch of monoterpenoid metabolic network, that converts pulegone, (a common precursor of menthol and menthofuran) to menthofuran, resulted in increased accumulation of menthol. This was achieved by making transgenics expressing antisense gene for *menthofuran synthase* (Mahmoud and Croteau 2001). Inhibition of specific steps in a metabolic pathway has also been attempted to allow accumulation of preceding intermediate. *Forsythia* plants produce lignans, such as matairesinol using pinoresinol as precursor. Pinoresinol is converted to matairesinol by pinoresinol/lariciresinol reductase (PLR) and secoisolariciresinol dehydrogenase. Down-regulation of *PLR* expression, using an RNAi construct led to a complete loss of matairesinol and a 20-fold accumulation of pinoresinol in its glucoside form, compared to the controls (Kim et al. 2009). In *P. somniferum*, reduction of codeinone reductase, an enzyme encoded by a multigene family was achieved by silencing the entire gene family using a chimeric small hairpin RNA construct. This led to accumulation of precursor alkaloid (*S*)-reticuline in the transgenic plants, at the expense of morphine, codeine, oripavine and thebaine (Allen et al. 2004). As an alternative, increased production of a secondary metabolite is also possible by engineering the regulatory mechanism of secondary metabolite biosynthesis. For instance, silencing of *DET1* regulatory gene using RNAi, in tomato fruits, resulted in increased apocarotenoid and flavonoid content (Davuluri et al. 2005). Overexpression of maize regulatory gene *leaf color* (*Lc*), in transgenic apples resulted in increased flavonoid content (Li et al. 2007). In maize suspension cells, overexpression of transcription factors *C1* and *R* led to increased accumulation of anthocyanins (Grotewold et al. 1998). Heterologous overexpression of maize *C1* and *R* genes in *Arabidopsis* resulted in increased pigmentation in normally pigmented tissues and induced pigmentation even in non-pigmented tissues (Lloyd et al. 1992). Overexpression of ORCA3, a transcription factor, in *C. roseus* results in upregulation of the genes of terpenoid indole alkaloid (TIA) pathway. ORCA3 directly interacts with the jasmonate and elicitor response element (JERE) in the upstream promoter region of *strictosidine synthase* (TIA pathway enzyme) and induces its increased expression. Transgenic suspension cells of *C. roseus*, simultaneously overexpressing ORCA3 and *G10H* (encoding a cytochrome P450 enzyme; not responsive to *Orca3* overexpression) led to threefold increase in accumulation of alkaloids (van der Fits and Memelink 2000).

Removal or reduction in quantity of toxic chemicals is also an important goal of metabolic engineering. Nornicotine, which is a precursor for a carcinogen, is produced by N-demethylation of nicotine. In transgenic tobacco plants, RNAi-induced silencing of CYP82E4 gene (encoding the enzyme that catalyzes this N-demethylation) was employed to suppress the production of nornicotine (Gavilano et al. 2006). *ZntA* gene of *Escherichia coli* encodes a lead/cadmium/zinc transporting ATPase. Ectopic expression of *E. coli ZntA* gene in *Arabidopsis* plants led to reduction in cellular levels of these heavy metals and improved resistance against lead and cadmium (Lee et al. 2003). In Coffee plants, *CaXMT1*, *CaMXMT1* (*theobromine synthase*) and *CaDXMT1* (*caffeine synthase*), enzymes successively add methyl groups to xanthosine converting it into caffeine. In sensitive individuals higher caffeine content could cause palpitations, increased blood pressure and insomnia. Transgenic coffee plants expressing RNAi constructs against these genes resulted in coffee with up to 70 % reduction in caffeine content (Ogita et al. 2003).

Sometimes metabolic engineering efforts may unpredictably yield novel compounds. Action of two multifunctional cytochrome P450 enzymes (CYPs) and a specific UDPG-glucosyltransferase catalyze the production of dhurrin from tyrosine. Overexpression of CYP79A1, first enzyme of the pathway, in *Arabidopsis* resulted in the formation of *p*-hydroxybenzylglucosinolates, which are normally not found in this plant species (Bak et al. 1999). In *E. californica*, RNAi-mediated suppression of berberine bridge forming enzyme leads to accumulation of reticuline which is a precursor of isoquinoline alkaloids. As an obvious outcome the products of this pathway, such as sanguinarine, were considerably reduced. However, laudanine, a methylated derivative of berberine accumulated in the transgenic plants (Fujii et al. 2007).

Exploring endophytes for production of plant secondary metabolites

Microbes that colonize host plant tissues without any apparent adverse effect (in contrast to pathogens), and survive in a mutualistic/commensal association are called endophytes. It is estimated that each plant may harbor one or more endophytic species (Tan and Zou 2001). The host plants, in their respective agro-geo-climatic zones provide unique niches to these microbes. In culture medium, outside their host plant species, these endophytes often produce bioactive compounds, which sometimes are the same as those produced by the host plant species. The power of this approach was first demonstrated by the discovery of taxol producing fungal endophyte *Taxomyces andreanae*, isolated from the host tree *Taxus brevifolia* Nutt. (Taxaceae) (Strobel et al. 1993). After this discovery, many other fungal endophytes isolated from various species of *Taxus* as well as from other trees were shown to produce taxanes (Pulici et al. 1996; Strobel et al. 1996; Bashyal et al. 1999; Wang et al. 2000a, b). Another plant metabolite, torreyanic acid, a potential anti-cancer agent, was found to be

produced from an endophytic fungus *Pestalotiopsis microspora* isolated from the endangered tree *Torreya taxifolia* Arn. (Taxaceae) (Lee et al. 1996). Endophyte isolated from *Podophyllum peltatum* L. (Berberidaceae) was reported to produce podophyllotoxin (Eyberger et al. 2006). *Dysoxylum binectariferum* (Roxb.) Hook.f. ex Bedd., (Meliaceae) is an endangered tree known for production of rohitukine, which is a precursor of flavopiridol (drug approved for treatment of chronic lymphocytic leukemia in EU) (Mahajan et al. 2014). An endophyte, *Fusarium proliferatum,* isolated from *D. binectariferum*, has been reported to produce rohitukine (Mohana Kumara et al. 2012). Microbes, due to their small generation time and high growth rates are desirable for industrial production of metabolites. Despite the discovery of high value plant secondary metabolites produced from endophytic fungi, to date there appears to be no report of commercial exploitation of these fungi for industrial scale production. It has been observed that after a few generations, the amounts of plant secondary metabolite produced by the endophytic microbe growing in culture medium, reduces to a great extent. For instance, a sharp attenuation in the production of camptothecin was noted from the first to seventh generation subculture of camptothecin-producing endophyte isolated from *Camptotheca acuminata* Decne. (Cornaceae) (Kusari et al. 2009). Horizontal transfer of genetic material (DNA or RNA) between the host plant and the endophytic microbe has been proposed to explain the production of phytochemicals by endophytes. Gene encoding 10-deacetylbaccatin-III-10-*O*-acetyl transferase was found to be present in the endophytic fungus *Cladosporium cladosporioides* MD2 isolated from *Taxus media* Rehder (Taxaceae). It shared 99 % identity with the homologous gene in host tree (*T. media*) and about 97 % identity with homologous genes in other species of *Taxus* (Zhang et al. 2009). In the endophytic fungus *P. microspora,* it has been observed that repeats of telomeric sequence 5′-TTAGGG-3′ are added to the termini of foreign transforming DNA and they replicate independently of the chromosomal DNA (Long et al. 1998). It is possible that these segments of DNA are lost or become silent during sub culturing, in the absence of any selective pressure. The endophytic microbe and host plant cells share an intimate and complex relationship. More research into this relationship and the biology of endophytic microbes may help to understand the phenomenon of attenuation in a better way.

Rewiring microbial biochemistry to produce plant secondary metabolites

An important component of elucidation of plant secondary metabolite pathways(s) is to clone and express the putative gene in a microbe (*E. coli* or *Saccharomyces cerevisiae*) and determine its biochemical activity on pure substrates so as to assign its role in the plant pathway. Co-expression of more than one gene of a pathway in a microbe results into a primitive metabolic cluster. For instance, an artificial curcuminoid biosynthetic pathway was constructed in *E. coli* by co-expressing phenylalanine ammonia-lyase (PAL) from the yeast *Rhodotorula rubra*, 4-coumaroyl:CoA ligase (4CL) from *L. erythrorhizon* and curcuminoid synthase (CUS) from rice (*Oryza sativa* L.; Poaceae), which resulted in the production of curcuminoids by the recombinant *E. coli* (Hwang et al. 2003). Recombinant *E. coli* cultures expressing 4-coumaroyl CoA ligase (4CL) from *A. thaliana* and stilbene synthase (STS) cloned from *Arachis hypogaea* L. (Fabaceae), converted the externally added precursor 4-coumaric acid to resveratrol (>100 mg/L) and externally added caffeic acid to piceatannol (>10 mg/L) (Watts et al. 2006).

Heterologous expression of a complete biosynthetic pathway of a complex plant secondary metabolite in a microbial host is considerably tough and tricky, compared to the above examples. Sometimes there may be missing links in a biosynthetic pathway; enzyme activities encoded by genes that have not yet been identified or cloned. Involvement of multiple enzymatic steps, which sometimes may not necessarily function in a linear fashion, makes cloning of multiple genes and their functional co-expression difficult in a microbial host. Even after overcoming most of these difficulties, and functionally expressing all the genes of a secondary metabolite biosynthetic pathway, the biggest challenge is optimization of enzyme activities to make the process economically feasible. For instance, biosynthesis of FDA approved, anti-cancer chemotherapeutic agent, paclitaxel (taxol) involves about 19 steps (Croteau et al. 2006). Reconstitution of first five committed steps of taxol biosynthesis in budding yeast for production of taxadien-5α-acetoxyl-10β-ol resulted in trace amounts of taxadien-5α-ol while taxadiene was produced only at a concentration of 1 mg/L (Dejong et al. 2006). Using regulatory proteins to inhibit competitive pathways, combinatorial biosynthesis and codon optimization of the cloned pathway genes, helped to increase the yield of taxadiene by 40 folds (Engels et al. 2008).

Lycopene, a bright red-colored carotenoid found in fruits and vegetables, is an antioxidant well known for its preventive activity against several types of cancers (Palozza et al. 2011; Takeshima et al. 2014). Heterologous expression of geranylgeranyl diphosphate synthase, phytoene synthase and phytoene desaturase in *E. coli* resulted in the production of lycopene (Bartley et al. 1999). An effective mutation screening method was used to identify targets for further increasing the production of lycopene in *Blakeslea trispora* fungus (Wang et al. 2013).

Advanced precursor of another FDA approved molecule—artemisinin, was produced in *S. cerevisiae* by heterologous reconstitution of a part of the artemisinin pathway from *A. annua*, involving five enzymes *CYP71AV1, CPR1, CYB5, ADH1* and *ALDH1*. Notably, the engineered yeast produced artemisinic acid to a concentration of 25 g/L. The artemisinic acid produced by fermentation can be chemically converted to artemisinin at a much lower price compared to extraction from plants (Ro et al. 2006; Paddon et al. 2013).

Microbes have thus immensely contributed in biotechnology of plant secondary metabolism by providing a model for its elucidation and as biosynthetic factories for production of phytochemicals.

Conclusion and Future perspectives

Bioprocessing of plant cultures holds great promise for production of phytochemicals. It provides an alternative method for production of phytochemicals on a large scale, in an economically viable and ecology friendly manner. Traditionally, brute-force methods have been used for selecting a better cell line for production of phytochemical of interest (Thomas et al. 2006). Combining bioprocessing with genetic engineering could help in making the tissue culture processes more productive. This however, requires a better understanding of the biosynthetic pathway and its regulation. It is known that certain pathways express better in suspension culture while others may not express at all (Berlin 1997; Chattopadhyay et al. 2002; Chiang and Abdullah 2007). This may be regulated by certain transcription factors that do not express well in undifferentiated cells (Xu et al. 2012; Patra et al. 2013). Alternatively, epigenetic changes like DNA methylation (Cazzonelli et al. 2010) or expression of certain microRNAs (Mahajan et al. 2011) may regulate the transcription of biosynthetic pathway enzymes. A detailed understanding of these regulatory mechanisms may help to rationally tinker with the secondary metabolite biosynthetic pathways. It is presumed that transport proteins might be playing a critical role in accumulation of secondary metabolites, at high concentration, in specialized cells or in a specific cell organelle (Brodelius and Pedersen 1993; Roytrakul and Verpoorte 2007). These proteins present another avenue for metabolic engineering of plant secondary metabolism. This needs to be done in conjunction with development of easier and faster plant transformation methods for medicinal plants. In cases where competitive pathways have to be inhibited, use of chemical inhibitors may be explored (Demain 1998; Sergeant et al. 2009; Craney et al. 2012). Alternatively, the use virus-induced gene silencing (VIGS) may be explored for silencing the targeted genes, to circumvent the need for development of elaborate transgenesis protocols (Huang et al. 2012). Construction of hybrid pathways, in engineered micro-organisms, using a combination of genes from different plant systems as well as other microbes, may be useful for optimizing/enhancing the yields of phytochemicals produced by the microbes. Use of genes from other systems that encode enzyme with analogous activities but are not sensitive to feedback inhibition may also be an option. Such innovative, synthetic biology approaches may also result in production of novel chemical scaffolds. Further, sharing of engineered microbial strains amongst researchers may aid the prospects of producing advanced/novel phytochemicals in microbes. Exploring the use of endophytes for phytochemicals has been criticized due to the unresolved mystery of attenuation of phytochemical production after a few generations (Kusari et al. 2009). However, there are reports where certain chemical activators were able to restore phytochemical production in otherwise attenuated endophytic cultures (Li et al. 1998). Use of such chemicals may also be explored. Analyzing the changes in genome sequence, epigenetic structure and transcription over successive generations may help to understand the reasons due to which endophytes loose the ability to produce phytochemicals after a few generations in culture. This may, in future, help to harness this resource of naturally engineered microbial strains that produce phytochemicals of interest, in a more meaningful manner. Further, bioinformatics tools for discovery, molecular understanding and methods for activation of silent or cryptic metabolic clusters are available for microbial species (Olano et al. 2014; Seyedsayamdost 2014), however, this remains a relatively unexplored area in plants. Discovery and activation of such silent metabolic clusters in plants, may result in production of novel phytochemicals.

Author contribution SGG conceived the idea, wrote the manuscript and prepared the figure. VM prepared the tables. YSB provided critical inputs during the preparation of this manuscript and carried out proofreading of the manuscript before submission.

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