Recent advances in medicinal plant biotechnology

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Medicinal plants are the most important source of life saving drugs for the majority of the world's population. Plant secondary metabolites are economically important as drugs, fragrances, pigments, food additives and pesticides. The biotechnological tools are important to select, multiply, improve and analyze medicinal plants. *In-vitro* production of secondary metabolites in plant cell suspension cultures has been reported from various medicinal plants and bioreactors are the key step towards commercial production of secondary metabolites by plant biotechnology. Genetic transformation is a powerful tool for enhancing the productivity of novel secondary metabolites; especially by *Agrobacterium tumefacians*. Combinatorial biosynthesis is another approach in the generation of novel natural products and for the production of rare and expensive natural products. DNA profiling techniques like DNA microarrays serve as suitable high throughput tools for the simultaneous analysis of multiple genes and analysis of gene expression that becomes necessary for providing clues about regulatory mechanism, biochemical pathways and broader cellular functions.

Keywords: Medicinal plants, biotechnology, combinatorial biosynthesis, DNA microarray, transgenic plants

Introduction

It is estimated that 70-80% of people worldwide rely chiefly on traditional, largely herbal, medicines to meet their primary healthcare needs[1]. The global demand for herbal medicine is not only large, but growing[1,2]. Various technologies have been adopted for enhancing bioactive molecules in medicinal plants[3]. Biotechnological tools are important for the multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformation[4]. It could also be harnessed for the production of secondary metabolites using plants as bioreactors. Advances in tissue culture, combined with improvement in genetic engineering techniques specifically transformation technology, have opened new avenues for high volume production of pharmaceuticals, nutraceuticals and other beneficial substances. Recent advances in the molecular biology, enzymology and fermentation technology of plant cell cultures suggest that these systems may become a viable source of important secondary metabolites. DNA manipulation is resulting in relatively large amounts of desired compounds produced by plants infected with an engineered virus, whereas transgenic plants can maintain constant levels of production of proteins without additional intervention. Large-scale use of plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemicals independent of plant availability[5,6]. Impact of specific engineering-related factors on cell suspension cultures has also been detailed[6]. Current developments in tissue culture technology indicate that transcription factors are efficient new molecular tools for plant metabolic engineering to increase the production of valuable compounds[7]. The approach to combine genes from different microorganisms for the production of new and interesting metabolites is known as combinatorial biosynthesis, which has emerged as a new tool in the generation of novel natural products, as well as for the production of rare and expensive natural products. There are several pharmaceuticals on the market that are highly expensive, due to the fact that these compounds are only found in rare plants and often in extreme low concentrations. Combinatorial biosynthetic strategies

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are expected to yield interesting alternatives in the near future. Combinatorial biosynthesis has been utilised for important classes of natural products, including alkaloids (vinblastine, vincristine), terpenoids (artemisinin, paclitaxel) and flavonoids. Conventional strategies for expression profiling such as northern blot, reverse northern blot, reverse transcriptase-polymerase chain reaction (RT-PCR), nuclease protection, enzyme-linked immunosorbent assay (ELISA), western blot, *in situ* hybridization and immunohistochemistry are optimized for single gene analysis. Although, it is possible to modify at least some of these techniques for multiplexing, the procedure becomes increasingly technically cumbersome. For genome wide expression analysis it is necessary to develop technologies having high degree of automation, since in any living organism thousands of genes and their products function in a complicated and orchestrated way. DNA microarrays have been developed in response to the need for a high-throughput, efficient and comprehensive strategy that can simultaneously measure all the genes, or a large defined subset encoded by a genome[8,9]. DNA microarrays are being used to study the transcriptional profile in various physiological and pathological conditions, leading to mining of novel genes and molecular markers for diagnosis, prediction or prognosis of those specific states[10]. This paper reviews the recent achievements and advances in the medicinal plant biotechnology.

Plant Tissue Culture

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Plant regeneration through somatic embryogenesis from stem, petiole and leaf explants of Indian chicory (*Cichorium intybus* L.) has been achieved[11,12]. The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, axillary buds and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors. The cultured cells and tissues can take several pathways. The pathways that lead to the production of true-to-type plants in large numbers are the preferred ones for commercial multiplication. The process of micropropagation is usually divided into several stages such as pre-propagation, initiation of explants, subculture of explants for proliferation, shooting and rooting and hardening. These stages are universally applicable in large-scale multiplication of plants. Micropropagation (*in vitro* propagation of axillary and/or adventitious buds as well as somatic embryos) is presently used as an advanced biotechnological system for the production of identical pathogen-free plants for agriculture and forestry[13]. Protocols for the cloning of some medicinal plants such as *Catharanthus roseus* (Apocynaceae), *Chlorophytum borivilianum* (Liliaceae), *Datura metel* (Solanaceae) and *Bacopa monnieri* (Scrophulariaceae) have been developed[14]. Moreover, *in vitro* flowering, *in vitro* fruiting and effective micropropagation protocols were studied in *Withania somnifera,* an antitumor medicinal plant using axillary buds implants[15]. A rapid micropropagation protocol was developed for *Hoslundia opposita* using nodal explants derived from mature trees[16]. The integrated approaches of plant culture systems will provide the basis for the future development of novel, safe, effective, and high-quality products for consumers.

Micropropagation by conventional techniques is typically a labour-intensive means of clonal propagation. Automation of micropropagation in bioreactors has been advanced by several authors as a possible way of reducing costs of micropropagation[17-19]. Bioreactors containing liquid media are used for large-scale growth of various tissues. The use of bioreactor for the micropropagation was first reported in 1981 for *Begonia*[20]. Since then it has proved applicable to many species including shoot, bulbs, microtubers, corms and somatic embryos[21]. Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. Functionally, plant culture bioreactors can be divided into two broad types: those in which the cultures are immersed partially or temporarily in the medium and those in which the cultures are continuously submerged. Bioreactors provide more precise control of the plant growth gaseous exchange, illumination, medium agitation, temperature and *p*H than the conventional culture vessels. Bioreactor-based propagation of plants can increase the rate of multiplication and growth of cultures and reduce the space, energy and labour requirements in commercial micropropagation. Three main classes of culture systems in bioreactors can be distinguished: (1) those producing biomass (cells or organogenic or embryogenic propagules, shoots or roots as the final product); (2) those producing metabolites and enzymes; and (3) biotransformation of exogenously

added metabolites (which may be precursors in the pathway). Less labour-intensive clonal propagation at lower cost through the use of modified air-lift, bubble column, bioreactors (a balloon-type bubble bioreactor) together with temporary immersion systems for the propagation of shoots, bud-clusters and somatic embryos have been developed. The bioreactor system has been applied for embryogenic and organogenic cultures of several plant species[22,23]. Significant amount of sanguinarine wase produced in cell suspension cultures of *Papaver somniferum* using bioreactors[24]. Ginseng root tissues, when cultured in a 20 tonne bioreactor, produced 500 mg/L/d of the saponin, which is considered as a very good yield[25]. Mass production of transformed *Panax ginseng* hairy roots in bioreactor has also been reported[26]. Much progress has been achieved in the recent past on optimization of these systems for the production and extraction of valuable medicinal plant ingredients such as ginsenosides and shikonin. Roots cultivated in bioreactors have been found to release medicinally active compounds, including the anticancer drug isolated from various *Taxus* species into the liquid media of the bioreactor, which may then be continuously extracted for pharmaceutical preparations[27]. Some of the recently produced bioactive secondary metabolites through plant tissue culture are presented in Table 1.

Combinatorial Biosynthesis

The basic concept of combinatorial biosynthesis is to combine metabolic pathways in different organisms at the genetic level, where genes from different microorganisms are combined for the production of new and interesting plant secondary metabolites (Fig. 1)[45]. From pharmaceutical point of view, hydroxylations and glycosylations are considered to be particularly useful bioconversions[38,39]. They can yield new drugs and existing drugs can be improved as to increased activity and decreased toxicity. Recent achievements with the polyketide biosynthesis from microorganisms, especially in *Streptomyces*, prove the potential of combinatorial biosynthesis[40-43]. Podophyllotoxin and paclitaxel are clear examples of pharmaceuticals that can only be produced through the isolation from plants. With regard to the production of podophyllotoxin it has been shown that plant cell cultures of *Linum flavum* can be used to convert deoxypodophyllotoxin, a major lignan of *Anthriscus sylvestris* into 6-methoxypodophyllotoxin[44]. The combination of the product of one

Table 1—Recently produced bioactive secondary metabolites from plant tissue cultures

Plant name	Active ingredient	Culture type	References
Cassia acutifolia	Anthraquinones	Suspension	28
Catharanthus roseus	Catharanthine	Suspension	29
Mentha arvensis	Terpenoid	Shoot	30
Nothapodytes foetida	Camptothecin	Callus	31
Podophyllum hexandrum	Podophyllotoxin	Suspension	32
Rhus javanica	Gallotannins	Root	33
Salvia fruticosa	Rosmarinic acid	Callus & suspension	34
Silybum marianum	Flavonolignan	Root	35
Taxus species	Taxol	Suspension	36
Withaina somnifera	Withaferin	Shoot	37

species and the enzymes of another species to yield a desired product forms a good example of combinatorial biosynthesis.

Combinatorial Biosynthesis of Terpenoids

Terpenoids represent a large and important class of natural products with more than 30,000 different structures. From pharmaceutical point of view the sesquiterpenoids are of high relevance. In this group artemisinin, gossypol and zingiberene are of great medicinal and economic interest. Terpenoids are biosynthesized via mevalonate (MVA) pathway or deoxyxylulose phosphate (DOXP) pathway. The MVA pathway has recently been expressed in *E. coli* harbouring the DOXP pathway, which led to an efficient production of the terpenoids, amorpha-4,11-diene and taxadiene[46-50].

Artemisinin

Artemisinin is an antimalarial drug isolated from *Artemisia annua* (Asteraceae). The selection of plants yielded varieties containing 0.5-1.16% of artemisinin in the aerial parts based on dry weight[51,52]. Alternatives could be the production via transgenic plants[53,54] or engineering the biosynthetic pathway into less complex host cells. This implies that the full elucidation of the biosynthetic pathway is required. Although several biosynthetic pathways have been postulated, until now only the genes encoding the enzymes for the synthesis of the first specific
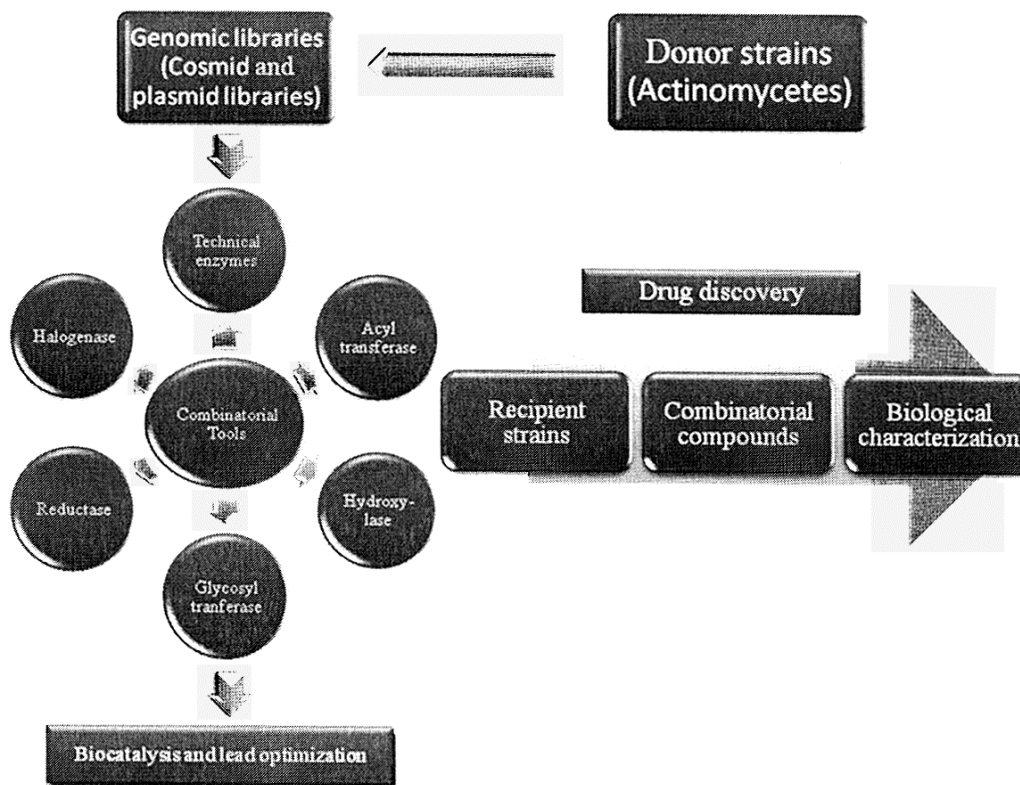
Fig. 1—Combinatorial biopsynthesis

intermediate amorpha-4,11-diene by amorphadiene synthase[55,56] and artemisinic acid by the cytochrome P450 enzyme, CYP71AV1[57] have been isolated and identified. The cDNA encoding amorphadiene synthase has been expressed in *E. coli* and characterized[58]. The recently discovered enzyme, CYP71AV1 was shown to catalyze the regioselective oxidation of amorpha-4, 11-diene into artemisinic alcohol. Besides this metabolic action, the enzyme also oxidizes the precursors artemisinic alcohol and artemisinic aldehyde yielding artemisinic acid[58].

Paclitaxel

Paclitaxel, mostly described by the tradename Taxol, is a diterpenoid that can be found in the bark and needles of different species of *Taxus*. The biosynthesis of paclitaxel starts with the cyclization step from geranylgeranyl diphosphate (GGDP) to taxadiene. Most of the 19 known enzymatic steps in the biosynthesis are related to hydroxylation and other oxygenation reactions of the taxadiene skeleton[59,60]. Several genes from different *Taxus* species that are responsible for steps in the biosynthesis and builing a basis for today's combinatorial biosynthesis in a heterologous microorganism have been isolated and

identified. Today, all the genes have been cloned into *E. coli* and activity screening confirmed the function of isolated enzymes[61-74]. The first intermediate, taxadiene can now be produced in *E. coli*. Co-expression of the taxadiene synthase from *Taxus brevifolia*[75] with a geranylgeranyl diphosphate synthase isolated from *Erwinia herbicola*[76], isopentenyl diphosphate synthase from *Schizosaccharomyces pombe*[77], and the endogenous deoxyxylulose 5-phosphate synthase from *E. coli* resulted in the production of 1.3 mg/L taxadiene in cell culture[78].

Carotenoids

Combinatorial biosynthesis of carotenoids in microorganisms has also been described[79]. The yeast, *Candida utilis,* has been engineered for the production of lycopene, ß-carotene, and astaxantin[80]. The production of carotenoids in a host requires the biosynthesis of the intermediate GGDP. *E. coli* produces the C15 precursor FDP for endogenous terpenoid molecules. The extension of the prenyl chain to C20 has been performed by the expression of CrtE gene encoding geranygeranyl diphosphate synthase from *Erwinia* sp.[81]. Prenyltransferase

catalyses the production of GGDP from FDP; the GGDP synthase encoding gene *gps* from *Archaeoglobus fulgidis* has been expressed as well. Expression of this gene is more efficient, because the enzyme catalyzes the three chain elongation reactions starting from C5 precursors to C20 molecule[82].

Combinatorial Biosynthesis of Alkaloids

Combinatorial biosynthesis has been reported for the alkaloids, vincristine, vinblastine, ajmaline and morphine from plants and for rebeccamycin and staurosporine from *Streptomyces albus*[83-85]. The morphine biosynthesis consists of 17 steps in *Papaver somniferum*. In the biosynthesis, a key intermediate, (S)-norcoclaurine is biosynthesized by condensation of dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA). The catalyzing enzyme (S)-norcoclaurine synthase has recently been identified from *Thalictrum flavum* and cloned in *E. coli*[86,87]. Further, key enzymatic steps towards (S)-reticuline include three NADPH oxidoreductases and cytochrome P450[88,89] and an acetyl-CoA dependent acetyltransferase[90,91]. Recently, the last step reducing codeine to morphine by codeinone reductase has been elucidated and the gene expressed in *E. coli* and insect cells[92].

Vinca Alkaloids

Vinblastine and vincristine, the monoterpenoid indole alkaloids from *Catharanthus roseus,* are used as antineoplastic drugs. Because of the high importance and the extreme low yields from plants (3 mg kg^{-1}) they could be considered as trace compounds[93]. *In vitro* application to improve alkaloid yield in *C. roseus* has been reviewed by many authors[94]. It is estimated that for the production of 3 kg of *Vinca* alkaloids, which is the annual need worldwide, around 300 tons of plant material has to be extracted. Production of *Vinca* alkaloids in plant cell cultures did not lead to a significant improvement and today it is accepted that biotechnological approaches in plant cell culturing may not provide an instant solution to this problem. The biosynthesis of vincristine and vinblastine is complex, from early phase starting from geraniol to strictosidine and for the late phase leading to the desired compounds. In the initial phase, tryptophan and secologanin as terpenoid precursors are condensed to form strictosidine as an important branching intermediate for other alkaloids. In this short part of the entire route seven enzymes and corresponding genes are involved, four of which have been cloned in *E. coli*[95,96].

Genetic Transformation Technology and Production of Transgenic Plants

Genetic transformation technology has been proved to be a powerful tool for the production of plants with desired traits in many crops[97,98]. It promises to overcome some of the substantial agronomic and environmental problems that have not been solved using conventional plant breeding programmes[99].

Agrobacterium and non-Agrobacterium Mediated Gene Transfer

Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most commonly used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants[100]. This soil bacterium possesses the natural ability to transform its host by delivering a well-defined DNA fragment, the transferred (T) DNA, of its tumour-inducing (Ti) plasmid into the host cell[101,102]. The rapid progress in the area of crop biotechnology is mainly because of the development of efficient regeneration and suitable *Agrobacterium*-mediated transformation protocols for different crop species[103,104]. Similar success could also be achieved in medicinal plants, which in turn could be used for the enhancement of secondary metabolite content. *Agrobacterium*-mediated genetic transformation of *Bacopa monniera* was standardized using the *A. tumefaciens* strain EHA105 that harboured the binary vector pBE2113 containing genes for glucuronidase (GUS) and neomycin phosphotransferase[105]. Transformation systems based on *A. tumefaciens* are well established for *Taxus* (yew)[106], *Echinacea*[107], *Scrophularia* (figwort)[108], *Digitalis* (foxglove)[109], *Thalictrum* (meadowrues)[110] and *Artemisia*[111]. An *A. tumefaciens*-mediated transformation system was developed for *Artemisia annua*. Analysis of artemisinin demonstrated that about 8-10 mg L^{-1} DW of artemisinin was detected in the transgenic plants regenerated from five shoot lines, which is about 2-3 times higher than the control[111].Thus, *Agrobacterium* transformation provides a method for routine genetic transformation of many important medicinal species. *Agrobacterium*-mediated gene transfer has the advantage for allowing stable integration of defined DNA into the plant genome that generally results in lower copy number, fewer rearrangements and more stablity of expression over generations than other transfection methods[112,113]. Recent studies have shown that virus-based vectors can be efficiently used for high transient expression of foreign proteins in

transfected plants and that non-*Agrobacterium* bacterial species can be used for the production of transgenic plants, laying the foundation for alternative tools for future plant biotechnology. Non-*Agrobacterium* species like *Rhizobium* sp. NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti,* are capable of genetically transforming different plant tissues and plant species[114].

Direct Gene Transfer
Generation of Transgenic Medicinal Plants by Particle Bombardment
Particle bombardment procedure was introduced in 1987, which involves the use of a modified shotgun to accelerate small (1-4 µm) diameter metal particles into plant cells at a velocity sufficient to penetrate the cell wall. There is no intrinsic limitation to the potential of particle bombardment since DNA is governed entirely by physical parameters. Different types of plant materials have been used as transformation targets including callus, cell suspension cultures and organised tissues such as immature embryos and meristems.

Cell suspension cultures were established from leaf explants of gentian (*Gentiana triflora × G scabra*) for generation of transgenic plants by particle bombardment[115]. Efficient transformation of the tropane alkaloid-producing medicinal plant, *Hyoscyamus muticus,* was also achieved by particle bombardment[116]. An efficient and stable transformation has been achieved in garlic plants (*Alium sativum*)[117]. The results indicate that biolistic transformation can lead to the transfer, expression and stable integration of a DNA fragment into chromosomal DNA. The relative simplicity of this system is a good recommendation for its future use in the production of genetically modified plants.

Generation of Transgenic Medicinal Plants by Electroporation
Electroporation uses brief pulses of high voltage electricity to induce the formation of transient pores in the membrane of the host cell. Such pores appear to act as passageways through which the naked DNA can enter the host cell. Exposure of cell suspension protoplasts of the woody medicinal plant, *Solatium dulcamara,* to a voltage of 250 to 1250 V cm^{-1} for three successive pulses, each of 10-50 µs duration, stimulated growth of protoplast-derived tissues[118]. Such tissues exhibited increased morphogenesis and required a shorter period in culture to exhibit this effect than tissues from untreated protoplasts.

Regenerated shoots also rooted more readily and developed more prolific root systems than shoots from untreated protoplasts. These observations have important implications for plant genetic manipulation and may have application in the recovery and rooting of shoots from tissues of woody species, normally considered recalcitrant in culture.

Generation of Transgenic Medicinal Plants by Chloroplast Transformation
Stable transformation of the chloroplast by inserting foreign genes into the chloroplast genome - was first achieved in the single cell green alga, *Chlamydomonas reinhardtii*[119] in 1988, soon to be followed by tobacco plant, and more recently, *Arabidopsis thaliana*. More than 40 transgenes have been stably integrated and expressed using the tobacco chloroplast genome to confer desired agronomic traits or express high levels of vaccine antigens and biopharmaceuticals[120]. Leaf discs are bombarded with plasmid constructs containing a selectable antibiotic resistance marker physically linked to the gene of interest, flanked by DNA for inserting into the correct site of the chloroplast genome. The antibiotic resistance marker most frequently used is the *aadA* gene encoding resistance for spectinomycin and streptomycin, driven by the promoter of the chloroplast encoded 16S rRNA.

Advantages of Chloroplast Transformation
1. There is no risk of the transgene being transmitted through pollen to plants.
2. Transgenes integrated into the chloroplast genome show very high levels of expression (up to 40% of the soluble protein in the cell may be the recombinant protein).
3. The transgene expression is not affected by gene silencing.
4. Chloroplasts express bacterial genes better than nuclei.
5. High level of transgene expression ensures a high level of mortality of the pests[121].

Pathway Engineering
Increasing the production of active phytochemical constituents is a well established target for genetic manipulation but presents some severe challenges. In particular, the metabolic pathways by which active compounds are biosynthesized are mostly poorly understood and relatively few genes for key enzymatic or regulatory steps have been isolated.

Nevertheless, there are examples of pathway engineering leading to improvements of potential value in the breeding of medicinal plants[122,123]. A recent article illustrating the challenges and opportunities of this approach describes a nine-fold enhancement in the production of a sedative compound, scopolamine, in hairy root cultures of *Hyoscyamus niger* (black henbane), brought about by simultaneously over expressing two genes encoding the rate limiting upstream and downstream biosynthetic enzymes[124]. Increase in the production of scopolamine in *A. belladona*, from the naturally occurring chemical precursor hyoscyamine, by transformation with the enzyme hyoscyamine 6 β-hydroxylase from *Hyoscyamus* is reported[125]. Preliminary progress has also been made towards engineering alkaloid production in *P. somniferum*[126]. A three-fold enhancement in the production of the putative antimalarial, anti-cancer agent, artemisinin, has been reported in transgenic *Artemisia* plants overexpressing farnesyl di phosphate synthase, the enzyme immediately preceding the first committed biosynthetic step[127,128]. New genomic approaches and efficient gene isolation methods applied to difficult secondary pathways in medicinal plant metabolism will undoubtedly expand the range and precision of manipulations via transgenesis, providing potentially superior material for the breeder.

Engineering Agronomic Traits in Medicinal Plants

There have been relatively few reports of biotechnology applied to abiotic stresses and other aspects of agronomic performance of medicinal plants, although the approach is considered to have great potential[129]. Just as resistance to herbicides, pests and diseases are the characters that have led the way in the introduction of transgenic crop species, so too have these characters been among the first targets for medicinal plant biotechnology. Transgenic *Atropa* plants resistant to the herbicides, bi-alaphosandglufosinate, has been described[130] and *Panax ginseng*, resistant to the herbicide, Basta, has been generated by transformation with the genes encoding enzyme phosphinothricin acetyl-transferase[131]. Resistant biotypes can also be a useful germplasm source for breeding. For instance, somatic hybridization, the fusion of somatic cells from tissue culture and atrazine selection, have been used to regenerate herbicide tolerant *Solanum nigrum* (blacknightshade)[132]. Transgenesis will assist in breeding varieties resistant to pathogens especially those causing fungal diseases. *Panax quinquefolium* (American ginseng) transformed with either a chitinase or thaumatin-like antifungal genes have been regenerated successfully[133, 134].

Bioanalytics and Metabolomics
Latest Hyphenated Techniques of Spectroscopic Analysis

The coupling of high performance liquid chromatography with nuclear magnetic resonance spectroscopy (LC-NMR) is one of the most powerful methods for the separation and structural elucidation of unknown compounds in mixtures. LC-NMR thus represents a potentially interesting complementary technique to LC-UV-MS in phytochemical analysis for the detailed on-line structural analysis of natural products. *Ligusticum chuanxiong* Hort., a plant that is frequently used in traditional Chinese medicine, has been studied using HPLC-coupled spectroscopic techniques such as HPLC-UV, HPLC-MS as well as HPLC-NMR[135,136].

Genomic Profiling of Medicinal Plants by DNA Microarray Technique

DNA microarray was developed in response to the need for a high-throughput, efficient and comprehensive strategy that can simultaneously measure all the genes or a large defined subset, encoded by a genome[137,138]. Several methodologies including differential display PCR, northern blots[139], quantitative PCR, serial analysis of gene expression (SAGE)[140,141] and TIGR Orthologous Gene Alignments (TOGA)[142, 143] are used along side microarrays as research tools. DNA microarray is an orderly arrangement of thousands of oligonucleotides or identified sequenced genes printed on an impermeable solid support, usually glass, silicon chips or nylon membrane. Novel technologies such as SAGE and DNA microarray allow rapid and detailed analysis of thousands of transcripts, providing a revolutionary approach to the investigation of gene expression. There are three main applications of DNA microarray:

(i) in pharmacodynamics for discovery of new diagnostic and prognostic indicators and biomarkers of therapeutic response; elucidation of molecular mechanism of action of an herb, its formulations or its phytochemical components and identification and validation of new molecular targets for herbal drug development.

(ii) in pharmacogenomics for prediction of potential side-effects of the herbal drug during preclinical activity and safety studies;

identification of genes involved in conferring drug sensitivity or resistance and prediction of patients most likely to benefit from the drug and use in general pharmacogenomic studies.

(iii) in pharmacognosy for correct botanical identification and authentication of crude plant materials as part of standardization and quality control.

The identification of genes modulated by compounds isolated from *Centella asiatica* with the help of gene microarray provides the basis for a molecular understanding of *Centella*'s bioactivity, and opportunities for the quantitative correlation of this activity with clinical effectiveness at molecular level[144]. Similarly, the antiproliferative activity of *Coptidis rhizoma*, a medicinal herb and its major component berberine was investigated in human pancreatic cancer cell lines and it was possible to identify common and distinct genes related to anti-proliferative activities[145]. High-density oligonucleotide microarrays have been used for pioneering studies on the multiple gene expression effects exhibited by *Ginkgo biloba* leaf extract EGb 761[146] and various studies on diets supplemented with *G. biloba* extract that have notable neuromodulatory effects *in vivo* and illustrates the utility of genome-wide expression monitoring to investigate the biological actions of complex extracts[147].

cDNA microarray analyses have shown that exposure of human breast cancer cells to a *Ginkgo* extract altered the expression of genes that are involved in the regulation of cell proliferation, cell differentiation or apoptosis, and that exposure of human bladder cancer cells to a *Ginkgo* extract produced an adaptive transcriptional response that augments antioxidant status and inhibits DNA damage[148]. Recent studies have highlighted the concurrent use of herbs which may mimic, magnify, or oppose the effect of drugs[149,150]. DNA microarray can be used for studying herb–drug interactions, and the mechanisms underlying these interactions.

DNA Microarray in Pharmacognosy

Use of an authentic herbal material is the first step of ensuring quality, safety and efficacy of herbal medicines. DNA polymorphism-based assays have been developed for the identification of herbal medicines[151,152]. Recently, microarrays have been applied for the DNA sequence-based identification of medicinal plants[153,154]. This includes quality control and standardization of the herbal drugs, identification

and validation of new targets, the profiling of on-target and off-target effects during the optimization of new therapeutic agents, understanding molecular mechanisms of action, structure–activity relationships[155] and the prediction of side-effects, and the discovery of diagnostic, prognostic, and pharmacodynamic biomarkers. Oligonucleotide probes specific for polymorphisms in the D2 and D3 regions of 26S rDNA gene of several *Fritillaria* species were designed and printed on the poly-lysine coated slides to prepare a DNA chip. Differentiation of the various *Fritillaria* species was accomplished based on hybridization of fluorescent labeled PCR products with the DNA chip. The results demonstrated the reliability of using DNA chips to identify different species of *Fritillaria*, and that the DNA chip technology can provide a rapid, high throughput tool for genotyping and plant species authentication[156]. Similarly, using fluorescence-labeled ITS2 sequences as probes, distinctive signals were obtained for the five medicinal species of *Dendrobium* listed in the Chinese Pharmacopoeia. It established that microarray was able to detect the presence of *D. nobile* in a Chinese medicinal formulation containing nine herbal components[157]. Phytomics, a technology platform for characterization of herbal compositions wherein, herbal bio response arrays (HBR Arrays) are used to determine bioactive constituents and biological activities of an herbal composition has recently been developed and patented[158].

cDNA-amplified fragment length polymorphism (AFLP) has been the functional-genomics tool of choice to study gene expression profiles related to the biosynthesis of secondary metabolites. cDNA-AFLP has the advantage as it does not require prior genomic data[159,160]. For most medicinal plants either limited or no information on genomic sequences is available, or cDNA libraries that could be used as a template for microarrays do not exist. cDNA-AFLP is done by cDNA synthesis from the tissue of choice and selective amplification. Genome wide profiling of transcript is done using softwares such as AFLP Quantarpro (Keygene, Wageningen, The Netherlands).

Marker Assisted Breeding

Marker assisted breeding involves the use of DNA markers linked with DNA sequences of interest. Inheritance pattern of sequences/traits can be confirmed even prior to expression using RFLP,

RAPD, AFLP and minisatellite markers[161,162]. A high content artimisinin producing plant variety "CIM-Arogya" was developed at CIMAP, Lucknow through marker assisted breeding. Selection of genotype with increased biomass led to selection of higher artimisinin yielding variety. An increase in artimisinin from 0.15% to 1.16% on dry weight basis was recorded in this variety[163]. Thus, marker assisted breeding could also be used in developing high secondary metabolite yielding plants.

Expressed Sequence Tags (ESTs) of Medicinal Plants

Monoterpene indole alkaloid (MIA) pathway genes were identified from random sequencing of *C. roseus* cDNA library which revealed 3655 unique ESTs, composed of 1142 clusters and 2513 singletons. Several novel MIA pathway candidate genes were identified by the cloning and functional characterization of loganic acid O-methyltransferase involved in secologanin biosynthesis. Biochemical pathways such as triterpene biosynthesis were also identified and its metabolite analysis revealed localization of oleanane-type triterpenes exclusively to the cuticular wax layer. The results illuminated, biochemical specialization of *Catharanthus* leaf epidermis for the production of multiple classes of metabolites[164]. Also, jasmonate-induced changes on the transcript and alkaloid profiles of tobacco BY-2 and *C. roseus* cell cultures have been monitored through the similar approach[165,166]. ESTs corresponding to 40 enzymes involved in the conversion of sucrose to sanguinarine were identified from elicitor induced cell culture of *Papaver somniferum*. Substantial increase in the level of RNA was observed in case of elicited cell culture as compared to control and the identified metabolites were sanguinarine, dihydrosanguinarine, methoxylated derivatives dihydrochelirubine and chelirubine, and the alkaloid pathway intermediates N-methylcoclaurine, N-methylstylopine, and protopine[167]. Similarly, cDNA library of *Artimisia annua* glandular trichome revealed the presence of many ESTs involved in isoprenoid biosynthesis such as enzymes from the methylerythritol phosphate pathway and the mevalonate pathway, amorpha-4,11-diene synthase and other sesquiterpene synthases, monoterpene synthases and two cDNAs showing high similarity to germacrene A synthases[168]. An inventory of hundreds of genes, potentially involved not only in alkaloid biosynthesis but also possibly in plant secondary metabolism in general, has been built.

Thereafter, large-scale functional analysis of genes from this inventory, potentially involved in plant secondary metabolism, was performed. This includes isolation, introduction and functional analysis of full-length open reading frames (FLORFs) in transgenic plant cells. Tools to improve and speed-up functional analysis of candidate genes in transgenic plant cells such as medium-throughput strategies for isolation of FLORFs, super-transformation of plant cells with reporter gene constructs, transient protoplast expression assays and microarray facilities, have been designed and their use validated.

Conclusion

The improved *in vitro* plant cell culture systems have the potential for commercial exploitation of secondary metabolites. Micropropagation, combined with *Agrobacterium* transformation, provides a method for routine genetic transformation of many important medicinal species. The production of secondary metabolites could be enhanced using bioreactors and has a tremendous potential for the large-scale synthesis of therapeutically active compounds in medicinal plants. During the past decade, remarkable progress in plant genetic-transformation technology has been witnessed. This rapid progress has resulted in constant flow of new and improved transformation protocols for many medicinal plant species. Genetic transformation may provide increased and efficient system for *in vitro* production of secondary metabolites. This review also highlights the possibilities for the use of bioconversion and combinatorial biosynthesis strategies for the production and development of plant natural products at different levels in biosynthetic pathways. There are several benefits of using microorganisms instead of plants or plant cell cultures, including their fast replication, low costs of cultivation, and the possibility for bioprocessing on an industrial scale. Microarray analysis of gene expression could be useful for elucidating the molecular mechanisms and networks underlying the complex pharmacological function of herbal extracts and mixtures. DNA microarrays have the potential for applications in different phases of herbal drug discovery and development. The tools described in this review would certainly be of increasing importance in the field of medicinal plant biotechnology research in near future.

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