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Review

Production of Active Compounds in Medicinal Plants: From Plant Tissue Culture to Biosynthesis

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ABSTRACT

Over past decades plant tissue culture has emerged as an alternative of whole plant cultivation in the production of valuable secondary metabolites. Adventitious roots culture of *Panax ginseng* and *Echinacea purpure* has reached the scale of 1−10 kL. Some molecular biological techniques, such as transgenic technology and genetic stability are increasingly used in the studies on plant tissue cultures. The studies on elicitors have deepened into the induction mechanism, including signal molecules, functional genes, and so on. More and more biological elicitors, such as *A. niger* and yeast are used to increase the active compounds in plant tissue cultures. We also discussed the application of synthetic biology in the studies on biosynthesis of artemisinin, paclitaxel, and tanshinon. The studies on active ingredients biosynthesis of medicinal plants provide unprecedented possibilities to achieve mass production of active ingredients. Plant tissue cultures can not only produce active ingredients but also as experimental materials for biosynthesis. In order to improve the contents of active compounds in medicinal plants, following aspects could be carried out gene interference or gene silencing, gene overexpression, combination with chemical synthesis, application of elicitors, and site-directed mutagenesis of the key enzymes.

Key words

biosynthesis; functional gene; medicinal plant tissue culture; secondary metabolites

1. Introduction

In recent decades, plant cell, tissue, and organ cultures have emerged as an alternative over whole plant cultivation for the production of secondary metabolites which are used as pharmaceuticals, flavours, fragrances, colouring agents, food additives, and agrochemicals (Paek et al, 2014). Plant tissue cultures can not only produce active ingredients but also as

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experimental materials for studies on synthetic biology. More recently, active ingredients biosynthesis of medicinal plants are being achieved through genetic and metabolic engineering approaches. Table 1 lists the production of active compounds through plant tissue culture or synthetic biology method. In this review, we summarized production of active compounds in medicinal plants by using plant tissue cultures and synthetic biology. We also discussed the relationship between plant tissue cultures and synthetic biology.

2. Production of active compounds through plant tissue culture

At present, cells, adventitious roots, hairy roots, shoots, and embryos have been successfully cultivated for the large scale production of secondary metabolites. Recent advances in plant cell, tissue, and organ culture research mainly focus on optimization of culture conditions, composition comparison, elicitors, transgenic technology, and genetic stability.

Table 1 Production of secondary metabolites in medicinal plants through bioengineering

Components	Secondary metabolites	Plants	Sources	References
terpenoids	taxuyunnanine c, taxol	*Taxus chinensis* (Pilger) Rehd.	cell, biosynthesis	Gao et al, 2010; Zhou et al, 2015; Zhang et al, 2010
	saponin	*Panax quinquefolius* L.; *A. Senticosus*; *P. ginseng*; *Bupleurum falcatum*	cell, hairy root, biosynthsis	Hao et al, 2010; Zhao et al, 2011; Tao et al, 2011; Balusamy et al, 2013; Moses et al, 2014
	glycyrrhizic acid	*Glycyrrhiza uralensis* Fisch.	hairy root	Zhang et al, 2011; Yang et al, 2014
	triterpenoid	*Codonopsis lanceolatae* (Sieb. et Zucc.) Trauv.	hairy root	Kim et al, 2011
	valerenic acid	*Valeriana officinalis* Linn.	hairy root	Torkamani et al, 2014
	saikosaponin	*Bupleurum chinense* DC.	adventitious root	Sun et al, 2013
	triptolide	*Tripterygii wilfordii* Hook.F.	adventitious root	Zhu et al, 2014a
	tanshinones	*Salvia miltiorrhiza* Bunge (Lamiaceae)	cell, hairy root, biosynthesis	Zhao et al, 2010; Guo et al, 2013
	artemisinin	*Artemisia annua* Linn	biosynthesis	Paddon et al, 2013
flavonoids	favonoids	*Ginkgo biloba* Linn; *Saussurea involucrate* Kar. et Kir. ex Maxim.	cell; hairy root	Hao et al, 2010; Hu, Han, and Zhou, 2011. Zhang et al, 2013; Qiao et al, 2011
	licochalcone a, total flavonoid	*G. uralensis*	hairy root	Zhang et al, 2011
alkaloids	alkaloid	*Fritillaria cirrhosa* D. Don.	cell	Wang et al, 2011
	total alkaloid, catharanthine, vindoline	*Catharanthus roseus* (L.) G. Don	cell, biosynthesis	Shukla et al, 2010; Moerkercke et al, 2015
	campothecin	*Campototheca acuminate* Decaisne	cell	Qi et al, 2010a; 2010b
	atractylodin	*Atractylodes lancea* (Thunb.) DC.	cell	Zhao et al, 2010
	ephedrine	*Ephedrae sinica* Staph	cell	Gandi et al, 2012
	tropane alkaloids	*Anisodus acutangulus* C. Y.	hairy root	Cao et al, 2014
	scopolamine, hyoscyamine	*Datura stramonium* Linn	hairy root	Sun et al, 2013
	vincamine	*C. roseus*	hairy root	Verma et al, 2014
	wilforine	*T. wilfordii*	adventitious root	Zhu, et al, 2014
	tricyclic aromatic quinines	*Aloe vera* (Linn.) N.L.	adventitious root	Lee et al, 2013
penylpropanoids	coumarins	*Angelica archangelica* L.	cell	Tomas et al, 2012
phenolic acids	phenolic acids, chlorogenic acid	*Eryngium planum* L.	cell	Kikowska et al, 2012
	rosmarinic acid	*S. miltorrhiza*	hairy root	Sheng and Chen, 2013;
	caffeic acid	*E. purpure*	adventitious root	Cui, 2013
quinones	acetylshikonin	*Arnebia euchroma* (Royle) Johnst; *Radix Arnebiae* Seu. Lithospermi	cell, hairy root	Baranek et al, 2012; Li et al, 2010; He et al, 2010
	anthroquinones	*Morinda officinalis* How.	hairy root	Zheng et al, 2014
	aloe emodin	*A. vera* Burman var. *chinensis* (Haw) Berg	adventitious root	Lee et al, 2013
	chrysophanol	*A. vera* *B. chinensis*	adventitious root	Lee et al, 2013
steroids	phytoecdysteroids	*Achyranthes bidentata* Blume	cell	Wang et al, 2013
	guggulsterone	*Commiphora wightii* (Arn.) Bhand.	cell	Suthar and Ramawat, 2010

2.1 Optimization of culture conditions

During medicinal plant tissue cultivation, a number of physical and chemical factors affect tissue growth and the synthesis of the target secondary metabolites, such as temperature, light, pH, electric, magnetic, electromagnetic radiation, mechanical force, ultrasonic, media types, media salt strength, inoculum density, carbon source, nitrogen source, and some trace metal ions in medium.

Generally, the culture temperature is 20−28 ^{o}C, and pH value of the medium is 5.6−6.0. Typically, sucrose is the best carbon source, for it has a heat labile nature and can be largely decomposed into *D*-glucose and *D*-fructose after autoclaved, only part of the sucrose left, which is more conducive absorption and utilization. In addition to providing a carbon skeleton and energy required in the medium, to some extent, carbon source can regulate osmotic pressure in medium. NAA, IBA, 2,4-D, and BA are the most common hormones. For *P. ginseng* tissue culture, IBA was found suitable for lateral root induction and growth (Jeong et al, 2009), and 2,4-D suitable for callus induction (Mercedes et al, 2002). Inorganic nitrogen, e.g., nitrate (NO_3^-) and ammonium (NH_4^+) in medium, is supplied with nitrate or ammonium salts. The relative proportions of nitrate and ammonium nitrogen levels have a significant impact on the cell division and differentiation of culture. The experimental results by Sivakumar et al showed that the lower ratio of ammonium nitrogen in the medium was more conducive to the synthesis of ginsenosides (Sivakumar et al, 2005). Zhang et al also reported that the lower nitrogen concentration in the medium was more conducive to the synthesis of polysaccharides (Zhang et al, 1996).

2.2 Comparison on chemical components in different tissue cultures

In recent years, chemical components in different tissue cultures were increasingly studied, including culture systems, cell lines, plant organs, culture mode and elicitors.

For examples, Chavan et al (2014) compared the content of phenolics in donor, DSO-derived and ISO-derived leaves using LC-MS, and results showed that the compounds identified were quite similar among them. In *Pseudostellaria heterophylla* (Miq.) Pax ex Pax et Hoffm. adventitious root culture, electrospray ionization tandem mass spectrometry (ESI–MS^n) analysis was performed to compare active chemical components of adventitious roots (AR) with filed roots, as a result, pseudostellarins A, C, D, and G were identified from AR (Wang et al, 2015). Furthermore, in *G. uralensis* adventitious root culture, ESI-MS^n analysis showed that two new flavonoids including (3*R*)-vestitol and glycyrol were identified in the salicylic acid treatment (Li et al, 2015).

2.3 Elicitors

Since the secondary metabolites in medicinal plants are at low levels, some active compounds are difficult to be isolated. Therefore, researchers try to induce the expression of specific genes in plant cells to increase the production of plant secondary metabolites by adding elicitors into culture medium. Lee et al (2013) studied the effects of salicylic acid, methyl jasmonate, ethylene on secondary metabolite accumulation in *A. vera* adventitious roots. It was found that the contents of aloe-emodin and chrysophanol were significantly increased and a total of 37 compounds could be produced when adventitious roots treated with salicylic acid.

2.4 Transgenic technology

Plant transgenic technology is a method of inducing variation of organisms heritable traits, by which exogenous genes transformed into the host plants are expressed after consolidation. The plantlets transformed with some genes can significantly improve the corresponding features of plants and produce high levels of active compounds or high biomass of plants. Agrobacterium-mediated method is not only the first transgenic method applied to plants, but also the most widely used method for plant transformation. In the plant tissue culture, plant transgenic technology is mainly applied in the establishment of hairy root culture and suspension cells.

Hairy roots transformed by Ri plasmid grow fast and contain the expression of complete metabolic pathways, which provide a broad prospect for the industrial production of secondary metabolites of medicinal plants. Furthermore, hairy roots provide a lot of plant materials for new drug screening because the biotransformation can produce many new compounds. At present, ginsenosides and berberine have been achieved commercial production by hairy root culture (Kim et al, 2011).

In the study on *Vitis amurensis* cell cultures (Aleynova et al, 2015), the effects of overexpression of other members of the CDPK multigene family (VaCPK9, VaCPK13, VaCPK21, and VaCPK29) on resveratrol accumulation and growth parameters of *V. amurensis* cell cultures were investigated. The results showed that overexpression of VaCPK29 could increase resveratrol content and fresh biomass accumulation in the four independently transformed cell lines of *V. amurensis* more than in the empty vector-transformed calli. Moreover, tryptophan decarboxylase (TDC) and strictosidine synthase (STR) genes from *C. roseus* have been successfully over-expressed in the rol gene integrated cell suspensions of *Echeveria setosa* V. minor (Priyanka et al, 2015).

2.5 Genetic stability

Plant tissue culture is a potential tool for rapid mass propagation of plants and has a great potential for controlling production of secondary metabolites. However the occurrence of cryptic genetic deficiencies as a result of somaclonal variation in regenerated plants can seriously limit the advantage of this system. Therefore, it is essential to establish genetic uniformity of tissue culture plantlets to ensure the quality of plantlets for its commercial value. Several strategies are available for detecting these variations

including inter simple sequence repeat (ISSR)-PCR, random amplified polymorphic DNA (RAPD), and flow cytometry.

ISSRs have been used in species with specific gene to investigate variability at the intraspecific level. The random amplified polymorphic DNA (RAPD) method is a PCR-based technique that amplifies random DNA fragments with the use of single short primers of arbitrary nucleotide sequence under low annealing conditions. The technique has been extensively used in species classification, genetic mapping and phylogeny (Liu et al, 2005; Rong and Yin, 2004). In the study of micropropagated *Ceropegia santapaui*, genetic stability was compared among direct shoot organogenesis (DSO)-derived plantlets, indirect shoot organogenesis (ISO)-derived plantlets and mother plants by ISSR and RAPD. The results showed that the gene of DSO-derived plantlets was similar to mother plants by both the methods of RAPD and ISSR, and ISSR fingerprints of ISO-derived plantlets showed low variation (Chavan et al, 2014). The application of flow cytometry in cell culture includes the following three aspects: (1) the flow cytometry instrument analysis and detection of cell programmed death (apoptosis). The technology has good speed of high precision accuracy. Huang et al (2014) studied the application of fluorescence microscopy and flow cytometry instrument mitochondria suspension cell activity and its physiological effects, and detected the programmed death rate of suspended cell; (2) flow cytometry was used to study the cell cycle kinetics and examine possible correlations between growth phases and contents of the target metabolites. Stancheva et al (2011) studied the flow cytometry analyses of the suspension's cell cycle kinetics. The results showed that proportions of cells in G_0/G_1 and S phases varied insignificantly (between 69%–76% and 9%–13%, respectively) during the cultivation, while the proportion of G_2/M-phase cells increased until the 8th day of cultivation when the exponential phase of cell growth ended. (3) Chromosome ploidy is analyzed using flow cytometry instrument. Heide developed a novel cell counting method based on a combination of cell-staining and automated confocal fluorescence microscopy. This method allowed us, for the first time, to determine the cell-specific productivity for plant cell suspension cultures (Heide, 2014).

In recent years, cells, adventitious roots, hairy roots, shoots, and embryos have been successfully cultivated for the large scale production of secondary metabolites. For example, adventitious roots of *P. ginseng* have reached the scale of 10 kL (Jeong et al, 2014), and adventitious roots of *Hypericum perforatum* L. reached 500 L (Murthy et al, 2014), adventitious roots of *Echinacea purpure* (Linn.) Moench reached 1000 L (Murthy et al, 2014), and the cultivation of somatic embryos of *Acanthopanax senticosus* (Rupr. Maxim.) Harms reached 500 L (Paek et al, 2014). Some molecular biology techniques are increasingly used in the studies on plant tissue cultures. However，there are still few examples of the commercialization of plant tissue cultures.

3. Application of elicitors

In spite of four decades of efforts, production of plant secondary metabolites by plant tissue culture technology is still facing many biological and biotechnological limitations. One of the major obstacles is the low yield of plant secondary metabolites in plant tissue cultures. Elicitors include chemicals or bio-factors from various sources that can active defensive system of the target living organism (Zhao, 2005). Since the major roles of plant secondary metabolites are to protect plants from biotic and abiotic stresses, elicitors can cause an array of defense reactions in plants, including accumulation of a range of plant defensive secondary metabolites in intact plants or in cell cultures. Therefore, elicitation has been used to improve the yield of plant secondary metabolites.

3.1 Elicitor signal transduction

The elicitors can be recognized by plant receptors which are located on the surface of the plasma membrane or endomembrane. The receptors are activated, and then in turn activate their effectors, such as ion channels, GTP binding proteins (G-proteins), and protein kinases and oxidative burst. Activated effectors can promote the synthesis of signaling molecules (salicylic acid, jasmonic acid, nitric oxide, etc.), which transfer the elicitor signals to downstream genes, and further amplify the elicitor signal to the biosynthesis of secondary metabolites (Blume et al, 2000).

3.1.1 Reactive oxygen species (ROS)

The generation of ROS is a common early response of plant cells to elicitor treatment (Zhao, 2005). There is an immediate cellular response to trigger plant defense signal, with increased accumulation of ROS such as hydrogen peroxide (H_2O_2), superoxide anions (O^{2-}) and hydroxyl free radicals (•OH). The ROS exerts various effects on plant defense responses, including defensive gene activation, as well as defensive compound induction. The plant antioxidant defense system can scavenge ROS by increasing the activities of the anti-oxidative enzymes (He et al, 2010). The plant antioxidant defense system can scavenge ROS by increasing the activities of the anti-oxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase. During the *Taxus* cell culture, with the addition of the crude extract of *Fusarium oxysporum*, the enzyme activities of SOD, POD, CAT changed greatly, and produced a lot of ROS; the contents of paclitaxel was four-fold of the control.

Though the mechanism about how ROS can regulate the metabolites induction is not clear, the increased accumulation of ROS, as the signal molecules, can mediate the thicken of plant cell wall, cell death, hypersensitive response, and expression of defense genes, which are the key signals of the induction of metabolic responses (Wang et al, 2015).

3.1.2 Nitric oxide (NO)

NO is a bioactive molecule that exerts a number of diverse signal functions in animal and plant cells (Beligni and Lamattina, 2000). NO can be generated in plants either non-enzymatically via light-mediated conversion of NO_2 by

carotenoids, or enzymatically from NO_2 by NADPH nitrate reductases (Neill et al, 2002). Studies showed that NO generation is a hallmark of plant cell death, the expression of defense genes, and plays a key role in oxidative burst and the synthesis of jasmonic acid. During the ginseng suspension culture, with the addition of NO donor sodium nitroprusside, the activities of oxidase NADPH were increased, and H_2O_2 were induced (Hu et al, 2003). Xu (2005) found that elicitor prepared from the cell walls of *Aspergillum niger* induces multiple responses of *H. perforatum* cells, including NO generation, jasmonic acid biosynthesis, and hypericin production. However, NO synthase inhibitors can effectively suppress the elicitor-induced jasmonic acid biosynthesis.

3.1.3 Ca^{2+} spiking

$[Ca^{2+}]_{cyt}$ spiking from extracellular and intracellular sources is also an early response that can be induced by elicitors, and it may mediate nearly all downstream elicitor-induced reactions (Rahimi, 2015). This dramatic elicitor-induced $[Ca^{2+}]_{cyt}$ spiking can directly regulate all defense gene expression or activates many intracellular processes through Ca^{2+} sensors, such as calmodulin, which can further activate Ca^{2+}/calmodulin-dependent protein kinase, membrane-bound enzymes, or transcription factors. For example, NAD(P)H oxidase, a major source of ROS production (such as H_2O_2 and O_2) is induced by $[Ca^{2+}]_{cyt}$ spiking. These ROS, protein kinase cascades, and lipid signaling messengers can further transfer and amplify elicitor signals to downstream reactions (Zhao, 2005).

3.1.4 Jasmonic acid (JA) and SA

Polyunsaturated fatty acids can produce signals, such as oxylipins including JA, MJ, and JA metabolites (Wasternack, 2007). JA and its related compounds have long been observed to be both an integral signal for biosynthesis of many plant secondary products and a transducer or mediator for elicitor signaling, leading to accumulation of plant secondary metabolites. Studies have shown that JA induces particular enzymes catalyzing biochemical reactions to produce low-molecular-weight defense compounds, for example, polyphenols, alkaloids, quinines, terpenoids, and polypeptides, in plants.

Salicylic acid is a signaling molecule that can usually be found very low concentrations in plants. The use of salicylic acid as an elicitor in plant tissue culture systems has been numerously reported. The production of this compound and its mechanism toward secondary metabolite biosynthesis is closely associated with the plant defense system. SA quickly accumulates at the site of infection during fungal elicitor treatment, and spreads to other parts of the plant to induce a wide range of defense responses, and induces gene expression related to biosynthesis and production of some classes of secondary metabolites in plants.

3.2 Classification of elicitors

On the basis of sources, elicitors can be divided into two types: biotic and abiotic elicitors.

3.2.1 Biotic elicitors

The biotic elicitors have biological origins from the pathogen or microbial cells, *viz* extracts of fungi, bacteria, viruses, and other microbial metabolites, especially the carbohydrate or polysaccharide fractions, which can be used to induce or stimulate the production of secondary metabolites in plant cell and tissue cultures (Patel et al, 2013). In *Taverniera cuneifolia* (Roth) roots culture, living microbial cultures, five fungal and five bacterial strains, were used as elicitors to improve glycyrrhizic acid content. Results showed that *Mucor hiemalis* stain can induce maximum 4.90 mg/g glycyrrhizic acid. Among bacterial elicitors, *Rhizobium leguminosarum* was the best strain for glycyrrhizic acid production (maximum 6.37 mg/g). They both were higher than the control (1.46 mg/g) and the MJ-treated group (2.57 mg/g). Endophtyic fungi are micro- organisms residing in internal tissues of living plants, which do not cause any immediate or overt negative effects. In recent years, the use of endophtyic fungi as elicitors to improve plant secondary metabolites has been widely applied. Gao et al (2011) found that treating *Euphorbia pekinensis* suspension cultures with endophytic fungal elicitor from *Fusarium sp*. E5 resulted in the biomass of culture was increased by 19.35%, whereas isoeuphpekinensin and euphol contents were 5.81 and 3.56 times of the control, respectively. The main components of the fungi and bacterial, such as polysaccharide and protein, also can be used as effective elicitors. Under the elicitation of protein elicitor isolated from the culture mycelia of *Tuber melanosporum*, the biosynthesis of ganoderic acids was significantly stimulated during *Ganoderma lucidum* fermentation (Zhu et al, 2014b).

3.2.2 Abiotic elicitors

In addition to the compounds from the process of plant defense reaction (phenylalanine, ethylene, salicylic acid, jasmonic acid, fatty acids, etc.), physical factors (such as UV, high temperature, low temperature, drought, electric field, magnetic field and pH) and chemical factors (such as heavy metals, inorganic salts, and some chemical synthesis elicitors) can be abiotic elicitors (Patel et al, 2013). They can promote the production of specific plant secondary metabolites related to the defense mechanisms of plants, and further induce the resistance of plants against pathogen and disease. Jasmonic acid and salicylic acid are important signaling molecules that have been used as exogenous elicitors to trigger secondary metabolism in plant cell and organ cultures. Jasmonic acid methylester, a common elicitor, has been successfully used in various plants, such as *P. ginseng*, *Bupleurum chinense* DC., *Stemona japonica* (BL.) Miq., *G. uralensis*, *P. quinquefolium* L, *A. senticosus* (Rupr. Maxim.) Harms, *Centella asiatica* (L.) Urban, and improved the metabolites accumulation in the plant cells. In addition, the precursors feeding, such as the addition of phenylalanin, and polyunsaturated fatty acids (linoleic and α-linolenic fatty acids), both can effectively elevate the accumulation of secondary metabolites in the cells.

During *Silybum marianum* adventitious root cultures, 100 μmol/L phenylalanine could be attributed to contents of taxifoline (0.143 mg/g DW), silydianin (0.117 mg/g DW) and silybin (0.031 mg/g DW), both of which were greatly rose up to 4.16, 2.44 and 1.58 fold of the control (0.03, 0.05, and 0.02 mg/g DW) after 72 h, respectively (Rahimi et al，2011). Linoleic acid is a substrate for jasmonic acid biosynthesis and can function as an elicitor for metabolite biosynthesis. Cultures supplemented with 5 μmol/L linolenic acid could enhance gymnemic acid content significantly (87.99 mg/g DW) which was 7.78 fold of the control (11.30 mg/g DW) (Praveena et al, 2015).

3.3 Genes

Elicitors can affect the chemical composition of plant tissues by influencing the expression of genes related to the secondary metabolite biosynthetic pathway. For instance, in cell cultures of *P. notoginseng*, three cDNA fragments of genes encoding squalene synthase (SQS), squalene epoxidase (SE) and cycloartenol synthase (CAS) related to triterpene biosynthesis were cloned. In HEJ or MJ treatment, up-regulation of SQS and SE genes and down-regulation of CAS gene were all observed (Hu et al, 2008). The polysaccharide fraction from endophytic *T. atroviride* D16 affected the chemical composition of *S. miltiorrhiza* hairy roots during cultivation, 1-deoxy-d-xylulose 5-phosphate reductoisomerase (DXR), geranylgeranyl diphosphate synthase (GGPPS), copalyldiphosphate synthase (CPS), and kaurene synthase-like (KSL) mRNA levels were observed to be gradually stimulated after treated by the polysaccharide fraction compared with control (Ming, 2013).

3.4 Elicitors enhanced triterpenoids production

Triterpenoid saponins are a diverse group of bioactive compounds containing one or more sugar moieties attached to hydrophobic triterpenoid aglycones. Although the biological role of triterpenoid saponins in plants remains largely unclear, these compounds have shown various medicinal properties, including anti-inflammatory, anti-cancerogenic and antiviral effects. Like glycyrrhizin in licorice and ginsenosides in ginseng, several of these compounds are major components of traditional herbal medicines described in Japanese Pharmacopoeia (Seki et al, 2015)

Flavonoids are polyphenolic compounds that are ubiquitously in plants. They are present in all dietary plants, like fruits and vegetables. Additionally, flavonoids are found in several medicinal plants, and herbal remedies and have been used in folk medicine around the world, especially in China. As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity in both *in vivo* and *in vitro* systems. The functionality in human health is supported by the ability of the flavonoids to induce human protective enzyme systems, and by a number of epidemiological studies suggesting protective effects against cardio-vascular diseases, cancers, and other age-related diseases. Here, we summarized some elicitors which can elevate triterpenoids (Table 2) and flavonoids (Table 3) production in the plant cells.

At present, the studies on elicitors have deepened into the induction mechanism, including signal molecules, functional genes and so on. More and more biological elicitors are used to increase the active compounds in plant tissue cultures. However, the study on composition of biotic elicitors is not deep, still remains in the mixture stage.

4. Production of active compounds by biosynthesis

The growth of many medicinal plants is influenced by environmental factors. Some rare medicinal plants grow slowly and are even difficult to be cultivated, for many active compounds in TCM are characterized with low contents, complex structure, unstability, difficulty in chemical synthesis. In recent years, the plant cell culture technology offers a viable strategy for the production of active compounds. However, the culture conditions of plant cell are strict and the growth of cells is slow.

Production of active compounds by the biotechnology have many advantages, including short production cycle, standard production system and stable quality and yield, which provide a new way to protect TCM resources. With the application of synthetic biology in the studies on biosynthesis of artemisinin, paclitaxel, tanshinon, and ginsenoside, synthetic biology has attracted widespread attention in sustainable use of TCM resources.

4.1 Biosynthesis of tanshinones

S. miltiorrhiza Bunge (Lamiaceae), known as tanshen or danshen. Tanshinones have been shown to exhibit a variety of biological activities, including antibiotic, anti-inflammatory, and antioxidant effects, as well as against heart diseases. Indeed, danshen preparations are in phase II clinical trials against the cardiovascular disease. Although the production of tanshinones is inducible in *S. miltiorrhiza* hairy roots, the current supply is dependent on field-grown plants, which are subject to variable yields, and genetic modification offers potentially increased yields in either systems.

Guo et al (2013) used a next-generation sequencing approach to identify six candidate CYP genes being coregulated with the diterpene synthase genes in both the rhizome and danshen hairy roots, and demonstrated that one of these, CYP76AH1, catalyzes a unique four-electron oxidation cascade on miltiradiene to produce ferruginol both *in vitro* and *in vivo*. They also built upon the previous establishment of miltiradiene production in *Saccharomyces cerevisiae*, with incorporation of CYP76AH1 and phyto-CYP reductase genes leading to heterologous production of ferruginol at 10.5 mg/L. As ferruginol has been found in many plants including danshen, the results and the approaches that were described here provide a solid foundation to further elucidate the biosynthesis of tanshinones and related diterpenoids.

Table 2 Enhancement of elicitors on triterpenoids production

Elicitors	Plant species	Results
Cell-free extracts of *Aspergillus niger*, *Saccharomyces cerevisiae*, *Agrobacterium rhizogenes*, *Bacillus subtilis*, and *Escherichia coli*	*Gymnema sylvestre* (Retz.) Schult	All elicitors showed a positive response to gymnemic acid, and *A. niger* had the highest response (11.2-fold).
Polysaccharide from *Fusarium oxysporium* Dzf17	*Dioscorea zingiberensis* C. H. Wright	At 20 mg/L on day 25 of culture after water extract was added to the medium, the cell dry weight was increased 1.34-fold, diosgenin 2.85-fold, and diosgenin 3.83-fold of control.
Fusarium sp. E5 mycelium	*Euphorbia pekinensis* Rupr.	The biomass was increased by 19.35%, whereas the isoeuphpekinensin and euphol contents were 5.81 and 3.56 times of the control, respectively; key enzymes were POD, CAT, and PAL activities
2-Hydroxyethyl jasmonate (HEJA)	*Panax notoginseng* (Burk.) F. H. Chen	HEJA could stimulate ginsenosides biosynthesis and change their heterogeneity more efficiently than methyl jasmonate; The total ginsenoside content and the Rb/Rg ratio were increased about 60 and 30% by HEJA of by MJA, respectively.
Germanium	*Panax ginseng*	Cultures supplemented with 60 mg/L organic germanium enhanced both fresh (565 ± 6) g and dry (44 ± 2) g biomass accumulations of adventitious roots and accumulation of Rb and Rg group of ginsenosides.
Yeast extract and methyl jasmonate	*Panax ginseng*	The maximum saponin production was 2.08% and was obtained from cultures treated with 500 μmol/L methyl jasmonate on the day of inoculation; This level was 28-fold of the control.
Oleic and linolenic acid	*Gymnema sylvestre*	Cultures supplemented with 5 μmol/L linolenic acid could enhance gymnemic acid content significantly (87.99 mg/g DW) which was 7.78 fold of the control (11.30 mg/g DW).
Oligosaccharides from *Fusarium*	*Dioscorea zingiberensis*	Both EOS and WOS significantly increased the activities of PAL, PPO and POD in the suspension cell and seedling cultures of *D. zingiberensis.*
Palmarumycin C13 from the *Berkleasmium* sp. Dzf12	*Dioscorea zingiberensis*	After palmarumycin C13 was added to the medium at a concentration of 60 mg/L, the diosgenin yield of the cultured plantlets reached its maximum of 6.44 mg/L and diosgenin yield of the cultured cells reached a maximum of 10.73 mg/L.
Linoleic and linolenic fatty acids	*Panax ginseng*	The biomass of 11.1 g/DW and maximal content of total ginsenoside (7.9 mg/g DW) were achieved after treated with 5 mol/L linolenic acid.
A. niger and *R. stolonifer*, yeast extract, salicylic acid, ascorbic acid, and eugenol	*Abrus precatorius* L.	Addition of the selected elicitor (*A. niger* and ascorbic acid) at optimized concentrations resulted in 24.6 g/L dry cell weight biomass and 53.62 mg/L glycyrrhizin, which was 5.22 times of the control.
Chitosan, methyl jasmonate, and yeast extract	*Glycyrrhiza glabra* L.	Methyl jasmonate of 100 μmol/L was more efficient in enhancing glycyrrhizin production up to almost 109 μg/g dry weight on day 5 of elicitation.

Table 3 Enhancement of elicitors on flavonoids

Elicitors	Plant species	Results
PEG8000 (2%) alone, yeast extract (YE) (0.1%) alone, or both of them	*Glycyrrhiza uralensis*	The total flavonoids were remarkably enhanced in the chi-transformed hairy roots after treated with the mixture of YE and PEG8000.
Copper sulphate	*Folium digitalis Lanatae*	The maximal content of flavonoids was induced after 24 hour elicitation (over 10 times of the control)
$Ce(NO_3)_3$, $AgNO_3$, $CdCl_2$, $CuCl_2$	*Tetrastigma triphyllum* (Gagnep.) W. T. Wang	Adding Ca^{2+} antagonists could be used to improve flavonoid production and cell growth jointly with metal elicitors during *in vitro* culture of *T. hemsleyanum* suspension cells.
Ultraviolet-B	*Jatropha carcas* L.	The combined levels of the three flavonoids in the cultures treated with the higher UV-B dose were 20-fold of the control.
Linoleic and linolenic fatty acids	*Panax ginseng*	The highest accumulation of flavonoids was observed at 5.0 mol/L for both linoleic and linolenic fatty acids.
Multi-walled carbon nanotubes	*Satureja khuzestanica*	The maximum contents of the total flavonoids (21.48 mg g/DW) and phenolics (35.74 mg g/DW) were obtained in the MWCNTs treatment at 100 μg/mL, 2.6 and 1.9 times of the control, respectively.

4.2 Biosynthesis of paclitaxel

Taxol and its structural analogs are among the most potent and commercially successful anticancer drugs. Taxol was first isolated from the barks of the Pacific yew tree, and early-stage production methods required sacrificing two to four fully grown trees to provide sufficient dosage for one patient. A semisynthetic route was later devised in which the biosynthetic intermediate baccatin III, isolated from plant sources, was chemically converted to Taxol. Although this approach and subsequent plant cell culture-based production have decreased the demand of the yew trees, production still depends on plant-based processes, with accompanying limitations on productivity and scalability (Ajikumar et al, 2010).

Recently, Zhou et al (2015) reported the co-culture of engineered bacteria strains, each of which contains the part of the pathway that it is best suited to host. For example, the synthetic pathway for the acetylated diol paclitaxel precursor were divided into two modules, expressed in either *S. cerevisiae* or *E. coli*, neither of which can produce the paclitaxel precursor. Stable co-culture in the same bioreactor was achieved by designing a mutualistic relationship between the two species in which a metabolic intermediate produced by *E. coli* was used and functionalized by yeast. This synthetic consortium produced 33 mg/L oxygenated taxanes, including a monoacetylated dioxygenated taxane.

4.3 Biosynthesis of artemisinin

The World Health Organization has recommended artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria caused by the parasite *Plasmodium falciparum*. Artemisinin is a sesquiterpene endoperoxide with potent antimalarial properties, produced by the plant *A. annua* Linn. However, the supply of plant-derived artemisinin is unstable, resulting in shortages and price fluctuations, complicating production planning by ACT manufacturers. A stable source of affordable artemisinin is required.

Paddon et al (2013) used synthetic biology to develop strains of *S. cerevisiae* for high-yielding biological production of artemisinic acid, a precursor of artemisinin. They demonstrated the complete biosynthetic pathway, including the discovery of a plant dehydrogenase and a second cytochrome that provide an efficient biosynthetic route to artemisinic acid, with fermentation of 25 g/L of artemisinic acid. Furthermore, they have developed a practical, efficient and scalable chemical process for the conversion of artemisinic acid to artemisinin using a chemical source of singlet oxygen, thus avoiding the need for specialized photochemical equipment. Because all intellectual property rights have been provided free of charge, this technology has the potential to increase provision of first-line antimalarial treatments to the developing countries at a reduced average annual price.

Terpenoids are the compounds most intensively studied in biosynthesis due to their wide pharmacological activities such as antitumor. Most studies on biosynthesis focused on functional genes, however, some transcription factors, resistance genes, and other regulation factors in plant metabolites pathway may also play an important role in the synthesis of secondary metabolites.

5. Conclusion

Based on the summary of the production of active ingredients in medicinal plants over the past decade, some progress on plant tissue culture and biosynthesis is as follows:

5.1 Plant tissue culture

Plant cells, adventitious roots, hairy roots, shoots and embryos have been successfully cultivated for the large scale production of secondary metabolites, such as adventitious roots of *P. ginseng* (10 kL), adventitious roots of *H. perforatum* (500 L), adventitious roots of *E. purpure* (1000 L) and somatic embryos of *A. senticosus* (500 L). Transgenic technology and genetic stability are increasingly used in the studies on plant tissue cultures.

5.2 Application of elicitors

Elicitors have been further studied in induction mechanism, including signal molecules, functional genes, and so on. More and more biological elicitors, such as A. niger and yeast are used to increase the active compounds in plant tissue cultures.

5.3 Biosynthesis

With the application of synthetic biology in the studies on biosynthesis of artemisinin, paclitaxel, tanshinon and ginsenoside, synthetic biology has attracted widespread attention in sustainable use of TCM resources.

5.4 Relationship between plant tissue culture and biosynthesis

Plant tissue cultures can not only produce active ingredients but also as experimental materials for biosynthesis. For example, plant tissue cultures were used in the studies on screening or verification of functional genes, because plant tissue cultures are easier to be obtained and operated than cultivated plants (Figure 1).

5.5 Future studies

Although great progress has been made in mass production of the active compounds of medicinal plants by the traditional Chinese medicine biological engineering technology, only several medicinal plants have reached the industrial-scale cultivation. The reason may be that the active compound contents in most medicinal plant cells and strains is low and instability. Therefore, we should reveal the

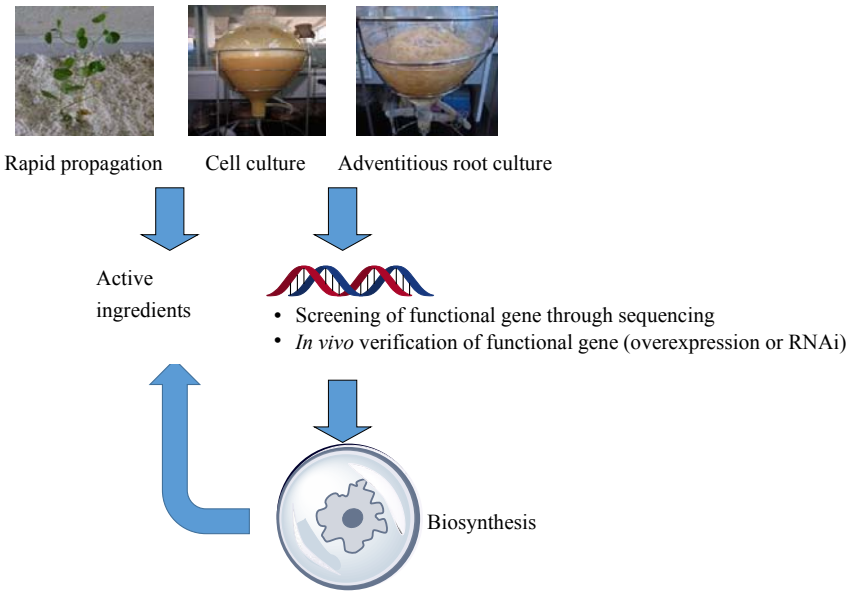

Figure 1 Full profile of medicinal plant bioengineering

regulatory mechanism of secondary metabolites at the molecular level to improve the active ingredients in plant tissue cultures or bacteria strains. Following aspects could be carried out: 1) gene interference or gene silencing; 2) gene overexpression; 3) combination with chemical synthesis; 4) application of elicitors; 5) site-directed mutagenesis of the key enzymes.

Conflict of interest statement

The authors declare no conflict of interest.

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