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# **Production of Useful Secondary Metabolites Through Regulation of Biosynthetic Pathway in Cell and Tissue Suspension Culture of Medicinal Plants**

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Hu Gaosheng and Jia Jingming

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/53038>

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## **1. Introduction**

Medicinal herbs played important roles in human history, from ancient times to now. They have been used for thousands of years to cure diseases, colorize clothes, adjust food taste and keep healthy. It was recorded that, 61% of currently used small molecular drugs are derived from or inspired by natural products from medicinal herbs [1]. However, to cure disease, we need to harvest medicinal herbs, and use part of their tissue for extraction, such as root, leaves, seeds, flowers and so on. That will directly cause problems for the reproduction of these herbs. It has also been noted that the natural habitats have been destroyed due to human activities. With the modern city under construction, the enlarging need for natural resources and the serious pollution, the environment has been never so tough for the growth of medicinal herbs. In another word, the naturally growing medicinal herbs can't fulfill the need of increasing market. Furthermore, the naturally grown medicinal herbs are also different from before, they are carrying more and more herbicide, insecticide and heavy metals, which will cause contamination to the extract and finally cause side effects. Besides, due to the complex structures of secondary metabolites, the chemical synthesis is proved to be cost-inefficient in most cases. Thus, how to produce enough medicinal herbal material in an appropriate manner becomes more and more urgent for the development of pharmaceutical industry all over the world.

In 1934, White proposed the theory of totipotency, and Steward proved the theory in 1952~1953 using carrot cells cultured in liquid media to regenerate whole plant. From then, the cell and tissue culture techniques are developed. As an alternative choice to produce active secondary metabolites, cell and tissue culture of medicinal herbs has obvious advantages:

1. The culture system doesn't need much field which can be used for crop growing;
2. The system is not limited by whether and season changes.
3. The secondary metabolism can be regulated to maximize the production of target compounds.
4. No herbicide and insecticide will be used during the maintaining of the system and therefore, the system is eco friendly.
5. Once the system is established, the content of useful compounds will be more stable than harvested herbs from different areas, which will facilitate the quality control.

Currently, hundreds of medicinal herbs have been used to establish different culture systems, such as callus culture, cell suspension culture, hairy root culture and adventitious root culture. Among these medicinal herbs, endangered species and herbs with positive anti-cancer, neuroprotection, anti-malaria, anti-oxidative activities attracted more attentions. With the development of molecular technology, more and more genetic information related to secondary metabolism becomes available, due to the hard work of generations of scientists from all over the world. After reviewing the research papers about secondary metabolism regulation, there are following strategies being developed to maximize the production of target compounds:

1. Over-expressing the key gene(s) involved in the biosynthetic pathway;
2. Blocking the competitive branches of biosynthesizing target compounds;
3. Blocking the degradation pathways or enhancing the transportation of target compounds;
4. Inhibiting the reproductive growth of plants and increase the biomass of vegetation growth, and increase the production of target compounds.
5. Introduce key genes into microbes and use combinatorial biosynthesis to produce target compounds or important intermediates.

Furthermore, plant cell culture techniques are playing more and more important roles in confirmation of gene function, expression, and contribution in the biosynthetic pathways of secondary metabolites. Plant cell and hairy root culture have become powerful tools in the genetic research fields. The reason is mainly because of the controlled growing environments (inoculated cells, light, pH, nutrient, shaking speed, temperature, treatments and pathogen-free etc.), stability and reproducibility of culture cells or organs.

Until now, many plant species have been used to establish different culture system, and useful compounds been targeted, among which, the accumulation of over 30 target compounds exceeded the content in wild plants. However, there are only shikonin (12% of dry weight) <sup>[2]</sup>, ginsenoside (in 20000 L scale) <sup>[3]</sup>, toxol (in 75000 L scale) <sup>[4]</sup> and berberine (13.2% of dry weight) <sup>[5]</sup> being produced using plant cell and tissue culture techniques in application scale, which is quite embarrassing. The bottle neck limiting the application of plant cell and tissue culture lies in the shortness of this technique: long culture period, low yield, high cost etc. However, there is another factor to be considered, that, in most medicinal plant, even the single compounds are being identified, few of them have potent activity against serious diseases, besides, in oriental medicines, most medicinal herbs are boiled in water together, following various

combinations, which made finding the effective compounds more difficult, and single compound(s) can't represent the curing effects. When we analyze the cultured callus, cells or tissues, we always can find the differences, compared with wild plant, which made it difficult to use the cultured cells or organs directly as wild plant materials. In one word, only if the target compound has high medicinal value, trying to increase the accumulation in culture system or combinatory biosynthesis microbes and finally purify the compound is worthy of the efforts. Without high medicinal value, the research can only be considered as a basic research solving mechanism related puzzles. Recently, combination of treatments are being developed in order to achieve high yield final products, such as elicitor treatment, repeated elicitor treatment, precursor feeding, over expression of key genes. In the future, the plant cell and organ culture system might have more potential in pharmaceutical industries.

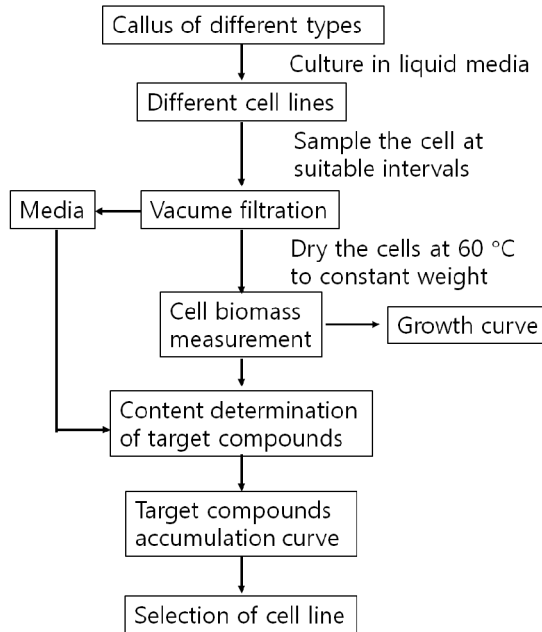
In the following contents, the establishment of different culture system will be introduced and recent progress of secondary metabolism regulation will be reviewed and discussed.

## **2. Establishment of culture systems**

To regulate the secondary metabolism of cells aiming at achieving maximized production of useful compounds, culture system should be established first. Here, establishment of three main culture systems will be introduced: cell suspension culture, hairy root culture and adventitious root culture. Three kinds of system are all liquid-form culture systems. The liquid form culture has advantages compared with solid form culture. Firstly, the contacting surface area of cells and media is much larger, and therefore, the nutrient will be much easier to be utilized by cells with higher efficiency. Secondly, compounds harmful for cell growth formed during the culture can be effectively diluted and avoid the inhibition of cell growth. Thirdly, in liquid culture system, the media is well mixed and dissolved oxygen can be monitored and controlled, finally, the scale-up of culture system is easier with the development of fermentation techniques.

### **2.1. Establishment of cell suspension culture.**

To establish cell suspension culture of a medicinal herb, firstly, the callus should be induced. During the callus induction, explants of plant origin should be surface sterilized and sliced into pieces about 0.5 cm<sup>3</sup> in clean bench, and inoculated on autoclaved solid basic media (MS, B5, N6, White etc.) supplemented with sucrose, hormones and agar. After the callus was induced, it should be sub-cultured. After sub-culture, usually, various types of callus can be found with different texture and color. Callus of various types should be introduced into liquid media (most of the time, after removal of agar, the formula of solid media can be used for liquid media preparation). Contents determination of active compounds should be carried out for cell line selection. In this step, cultured cells of different types should be sampled at each growth stage, and the cell samples will be subjected to biomass measurement and content determination as shown in Fig. 1, because cell of different types might be growing at different speed, and the maximal yield of target compounds can also be different when collected at same culture time.

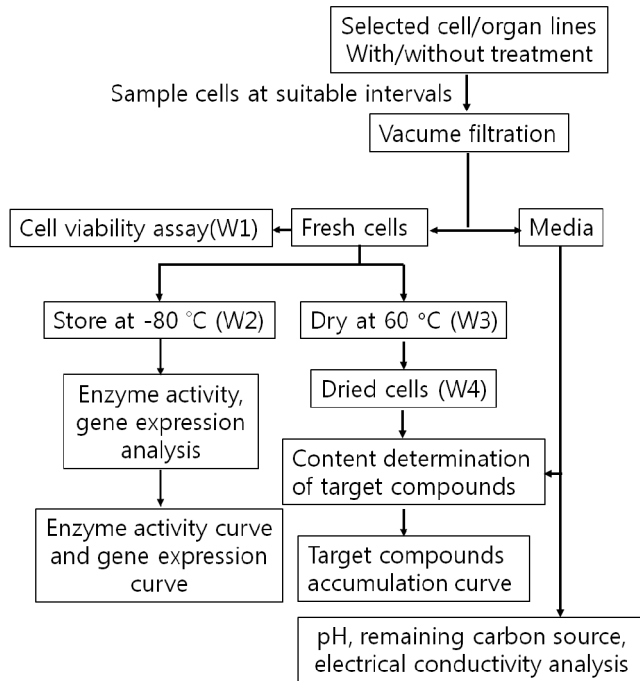


**Figure 1.** Flow chart of cell line selection

Once the cell line is selected, investigation on cell growth, cell viability, pH changing, carbon source consumption, enzyme activity, gene expression, target compounds accumulation can be carried out following the flow chat shown in Fig. 2. The total dry weight (DW) is calculated following the equation:  $DW=W4 \times (W1+W2+W3)/W3$ . To be noticed is that, the DW is a very important index, which will be used in growth curve, target compound yield, enzyme activity curve, gene expression curve preparations. In some cases, target compounds can be secreted into media, and therefore, the content determination of target compounds in liquid media should also be taken into consideration. The compounds detected in media and in dried cells should be summed to represent the compound producing ability.

## 2.2. Establishment of hairy root suspension culture.

For many medicinal herbs, roots are the medicinal part used for extraction, such as *Panax giseng*, *Panax notogiseng*, *Coptis chinensis*, *Savia miltiorrhiza* and so on. Furthermore, it has also been found that, in differentiated tissues, secondary metabolites accumulation is usually higher than callus and cells without differentiation. Therefore, hairy root can be an effective candidate culture form.



**Figure 2.** Flow chart of different investigation using cell sample and media

*Agrobacterium rhizogenes*, belonging to the family of Rhizobitaceae and genus of *Agrobacterium*, is a gram-negative soil bacterium. It has flagellum and can swim. The victim host can be most dicotylendous plants, few monocotylendous and specific gymnosperms plants, hairy root will be formed on the infected sites.

It has been demonstrated that, outside of chromosome, huge covalent circular double stranded DNA exists, and the size is between 180 and 250 kb, which is named *Ri* (Root inducing) plasmid. *Ri* plasmid has two major parts, virulent region (*Vir*) and transfer region (T-DNA). With the assistance of *Vir* region, T-DNA can be transferred to host plant, and integrated to host plant chromosome. In T-DNA, there is a gene called opine synthase with a eukaryotic promoter. After the integration happens, opine synthase promoter started to work and plant cell will be forced to synthesize a specific non-coding amino acid, opine, which will be used as the only carbon source for infected cells. The infected cells grow vigorously, and the phenotype is hairy root. Following are hairy root induction protocol:

#### 1. Preparation of sterile explants

Explants can be obtained from seedlings germinated from sterile seeds of medicinal herbs. Young tissues (leaf, stem, shoot) of plants collected from field can also be used as explants

after surface sterilization. After sterilization, the explants can be inoculated on basic solid media supplemented with (50-200mM) acetosyringone.

## 2. Preparation of *A. rhizogenes*

Bacterium stock stored at -80°C deep freezer can be streaked on solid YEP media, and single colony is selected for culture in liquid YEP media for 16 h in dark. Culture condition is 28°C and shaking speed is 200rpm. After the OD<sub>600</sub> reached 0.6-1.0, the bacterium culture can be used for infection.

## 3. Infection

Centrifuge the bacterium culture obtained above at 4000 rpm for 30 min at 4 °C, and resuspend the culture in same volume YEP media supplemented with 50-200uM acetosyringone. Immerse the wound sterile explants in resuspended Agrobacterium culture for 10 min and dry its surface on filter paper to remove excess agrobacterium. The wound is essential for successful hairy root infection, and usually there are a few wounding methods using sterile needles and sharp knife. The wound doesn't need to be serious, slight wound exposing inside tissue would be enough for infection to happen. Inoculate the infected tissue in basic media supplemented with acetosyringone.

## 4. Co-culture

Incubate the petri-dish in dark at 20-22°C for 2-5 days until obvious bacterium growing is visible.

## 5. Wash

Wash the infected explants from step 4 with 75mg/L hygromycin and carbonicillin water solution for at least 5 times. Vortex the tissue to remove the excess bacterium.

## 6. Hairy root growing

After washing, the tissue can be inoculated on solid media with acetosyringone and carbonicillin. It will take about two weeks for the hairy root growing until visible. During this process, once agrobacterium colony is visible on solid media, the tissue should be washed again following step 4.

## 7. Hairy root culture

Once hairy root is germinated from infected sites, the root can be separated from explants and culture on solid media until enough biomass is obtained. Then the hairy root will be inoculated in liquid media incubated in shaking incubator at 25 °C and at about 130 rpm.

### **2.3. Establishment of adventitious root suspension culture.**

Adventitious root is another model culture system, which can produce identical components compared with wild growing plants. Compared with hairy root culture, adventitious root will not produce opine, grow slower and require no genetic transformation techniques. Therefore,

for the directly further use of cultured roots, this system provides safer product, after all, opine is a harmful compound for human beings. Adventitious root can be induced from sterile explants, callus and seedlings of plant. The hormone used most widely is IBA. After induction, the callus or explants together with adventitious root can be inoculated into liquid media and cultured in shaking flask or bioreactors. Until now, the most famous application is the adventitious root culture of *Panax ginseng*, that, the culture system has been scaled up to 10 tons and the total saponin yield reached 500mg/L/day<sup>[6]</sup>.

#### 2.4. Application of bioreactors.

The application of bioreactor is one of the prerequisites for industrialization of plant cell culture. Therefore, bioreactor design specific for plant cell is important for the production of secondary metabolites using cell culture techniques. Based on the structure, plant cell bioreactor can mainly be divided as 5 types: mechanical stirring, air-lifting, bubbling, nutrient mist and temporary immersion bioreactors. These bioreactors have some characteristics in common that they can provide well mixed media and sterile air. Different from shaking flask, that the shaking will allow the media contact with air in the flask continuously, the media in bioreactor should be stirred or lifted by air bubbles, or mixed with air and then provide to cultured cells or tissues. Therefore, provision of air becomes very important for bioreactors. In bioreactors, there are several sensors monitoring the pH changes, temperature, dissolved oxygen (DO) and generated bubbles, the automated bioreactor can control these indexes following the order set by the operators.

Usually, the mechanical stirring bioreactor can produce highest DO index, however, it is not used widely in plant cell and tissue culture, because they are sensitive to shear force. In case of air-lift and bubbling bioreactors, they have similar structures and used quite often in plant cell and tissue culture. In hairy root culture, the bioreactors are always equipped with layers of meshes made of stainless steel, to provide attaching area for hairy roots. The nutrient mist and temporary immersion bioreactors have similar characteristics and they are both used for tissue culture, such like hairy root, adventitious root, shoots, bud clusters. They are all composed of two parts, media storage part and tissue culture part. After mixing of media and sterile air, for nutrient mist bioreactor, the media is sprayed through atomizer into tiny droplets on the surface of cultured tissues, and for temporary immersion bioreactor, the media is pumped to the culture part and maintain for short time and then the media is recovered to storage tank.

There are successful examples for cell and tissue culture of medicinal herbs to produce useful secondary metabolites. *Digitalis purpurea* cell line was cultured in airlift bioreactors, and the yield of methyl isopropyl hydroxyl digitoxin reached 430 mg/L; *Phalaenopsis aphrodita* protocorm like body culture in temporary immersion bioreactor for micro-propagation<sup>[7]</sup>; *Panax ginseng* adventitious root culture in bollon type bubbling bioreactor at scale of 20 tons<sup>[3]</sup>. All these achievement demonstrated the industrial potential of the application of bioreactor in producing secondary metabolites with high medicinal value.

### 3. Biosynthetic pathways of secondary metabolites

#### 3.1. Biosynthetic pathway introduction of secondary metabolites in medicinal plants

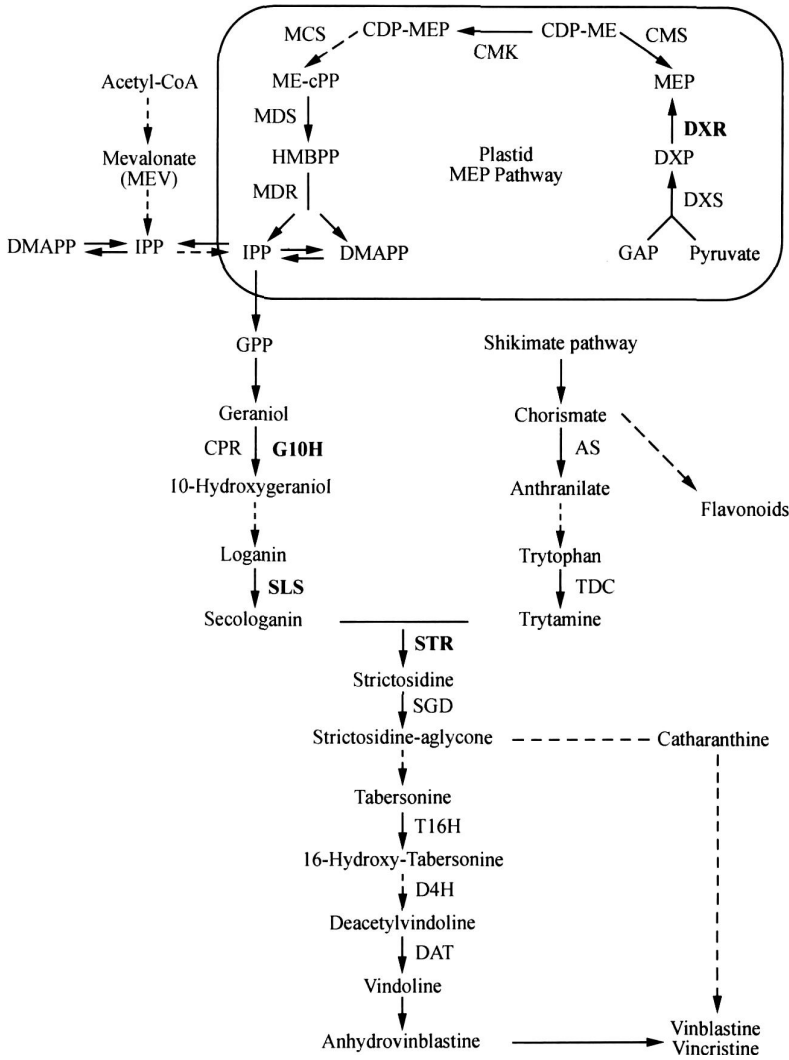
During long period evolution, plants struggling to survive gradually gain the ability to synthesize various kinds of secondary metabolites with bioactivities. These compounds played important role in defencing insects, herbivores, microbial pathogens, competing with other plants, and facilitating pollination and reproduction. Based on the structures, the second metabolites can be classified into alkaloids, flavonoids, phenylpropanoids, quinones, terpenoids, steroids, tannins and proteins. These compounds are biosynthesized through series enzyme catalyzed reactions using simple building blocks in different ways. There are several main biosynthetic pathways in plants, including shikimic acid pathway (phenylpropanoids), mevalonic acid pathway (quinones), 2-C-methyl-D-erythritol-4-phosphate pathway (quinones), amino acid pathway (alkaloids), acetate-malonate pathway (fatty acid, phenols and quinones) and combined pathways (flavonoids). These biosynthetic pathways are well reviewed and discussed in publication [8], and the current determined steps in biosynthesis pathways are also centralized and documented in KEGG [9] website, therefore, won't be further introduced in this chapter.

#### 3.2. Molecular cloning and characterization of key enzymes involved in specific biosynthetic pathway.

Gene cloning, transformation and regulation have achieved significant progress in biosynthetic pathway of secondary metabolites. The biosynthetic pathway elucidation (Fig. 3) and gene regulation research of vinblastin and vincristine, anticancer compounds from *Catharanthus roseus*, is a representative example. Due to the low contents of these two compounds in naturally growing plant, it becomes important to clone and regulate expression of key genes (DXR, SLS, G10H, STR) involved in the pathway, in order to achieve high yield of these two compounds in invitro culture systems, which will be based on well understanding of terpenoid indole alkaloids biosynthesis in *C. roseus* [10].

As to the approaches applied in the cloning of genes involved in secondary metabolites biosynthetic pathway, there are methods based on Polymerase chain reaction (PCR) and library construction. The former methods include Rapid amplification of cDNA ends PCR (RACE PCR) and RT-PCR. However, these methods need at least partial gene sequence information, or gene sequence from other plant species to synthesize degenerate primers. If there are no reported reference sequences, these methods will not be able to work well. Under this situation, library based method can be selected for gene cloning, however, the library based methods are non specific enough, like cDNA library, BAC library. That's because of the low ratio of genes involved in secondary metabolism in total expressed mRNAs. As the progress of genomics, functional genomics provided us useful methods in cloning the specific genes involved in the secondary metabolism, such as Subtractive hybridization 'Differential screening' Microarray assay and Serial Analysis of Gene





**Figure 3.** Biosynthetic pathway of terpenoid indole alkaloids in *C. roseus* (Key enzymes are shown in bold)

Expression. Functional genomics focused on the selection of treatment responsive genes and to clone genes involved in the biosynthetic pathways, the treatments need to be specific enough to result in the improvement of target secondary metabolites.

### 3.3. Elicitors and signaling pathways

Elicitors are substances that can trigger the hypersensitive reaction in treated plant cells. Due to the effective up-regulation of genes expression, and further activation of secondary

metabolism, and the improvement effects of secondary metabolites accumulation, elicitors are used widely in medicinal plant cell and tissue culture to maximize the production of target compounds.

Elicitors can be divided into biotic and abiotic elicitors. The former one includes fugal polysaccharides, proteins, cell debris and conidium. These kinds of elicitors are made from cultured fungi and some species are specific for specific kinds of secondary metabolites accumulation, for example, *Armillaria mellen* elicitor for alkaloids accumulation; *Verticillium dahlia* for phenolic compounds accumulation; *Botrytis sp* for terpenoids accumulation, oligosaccharides for saponins accumulation and yeast extract for flavonoids accumulation. As to abiotic elicitors, heavy metals ions, rare metal ions, UV lights, osmotic stress and even sonication have all been reported to have positive effects towards improvement of secondary metabolites. Comparing the application potential, biotic elicitor attracted more attention due to their advantages of low cost, little side effects, strong elicitation effects and easy manipulation.

When using elicitors, the main optimizing characters are treatment time, concentration and selection of elicitor kinds. In plant cell/organ culture, it is believed that, treat cells/organs with elicitors at the stabilization stage can increase the accumulation of secondary metabolites significantly. However, there are also reports that treat cells at the beginning of culture, which also improved the final product yield remarkably. In this case, there is possibility identifying key intermediates involved in the biosynthetic pathway. As to the concentration, too high concentration treatment can result in decrease of biomass accumulation and even the content of secondary metabolites can be improved, however, the total yield will still not be maximized. Therefore, to use an elicitor, the conflict between biomass accumulation and secondary metabolite accumulation should be balanced and the optimization should be carried out. Recently, a strategy of repeated elicitation has been developed, and the treatment can result in the increase of target compounds accumulation as well as related enzyme activity and gene expression more than one time, and therefore, attracts attention in the field of secondary metabolism regulation aiming at maximizing the yield of compound of interest.

### 3.4. Precursor feeding

Precursors are compounds existed in upstream of target compounds in biosynthetic pathway. Most intermediates can be used as precursors. Upstream precursors are converted into down-stream compounds after specific enzyme catalysis, the concentration of precursors determines the reaction speed. At higher concentration, the reaction speed is usually higher than that when precursor concentration is lower. To improve the yield of secondary metabolites in plant cell and tissue culture, precursor feeding is an effective approach. However, precursor can inhibit cell growth and enzyme activity if concentration is too high. In some cases, high concentration of intermediates can even be toxic for cells. Originally, in cells, these intermediates are all maintained in very low concentration, and converted to down-stream compounds quickly. Therefore, for one specific plant species, precursor concentration should be carefully adjusted, compared and optimized.

The precursor feeding experiment can also be used as a powerful tool for the elucidation of biosynthetic pathways. Isotope labeled precursors can be fed in the media, and as the cell growing and biosynthesis going on, secondary metabolites synthesized can be isotope labeled at different positions. After harvesting the cells, purifying the compounds and elucidating their structures, the biosynthetic origin can be concluded. This method is classic but also most authentic method in elucidating the biosynthetic origin of a specific compound. However, the disadvantages are also obvious, first of all, the precursors are isotope labeled and is dangerous to handle; secondly, the purification of isotope labeled end product requires lots of cells; thirdly, some intermediates are maintained in very low concentration in living plant cells, which will makes the purification of these intermediates very difficult. Therefore, if the isotope labeled precursor feeding combine with elicitor treatment or specific enzyme inhibitors treatment, the problems might be solved.

### **3.5. Application of specific enzyme inhibitors in regulating the biosynthetic pathway**

In plant cells, all secondary metabolites are derived from glucose that is biosynthesized during photosynthesis. In most medicinal plants, and even model plants, there are several kinds of secondary metabolites accumulated. For example, there are about 15 flavonoids, 50 terpenoids, 15 fatty acid, 20 phenylpropanoids, and 35 glucosinolates in *A. thaliana* [11].

Different kinds of compounds are competitively related with each other. To improve the accumulation of one specific kind of compounds or blocking the other main biosynthetic branches, genetic transformation method is ideal for the purpose. However, the transformation method of most medicinal plants has not been established. On the other hand, elicitor treatment is not specific, even the treatment can result in the improvement of secondary metabolites accumulation. Therefore, both methods can't provide specific evidence for contribution of single gene in plant secondary metabolites biosynthetic pathway. Under these circumstances, how to study the contribution of specific gene(s) involved in the biosynthesis pathway to the production of target compounds?

If we treat the cells with enzyme specific inhibitors, the enzyme activity will be specifically inhibited, and result in the corresponding consequences. After content determination of target compounds and enzyme activity assay, correlation analysis between enzyme activity and target compounds accumulation can be carried out, and therefore get to know the contribution of specific enzyme in the biosynthetic pathway of target compounds. This method can also be used to identify key enzymes or rate-limiting steps in the pathway. The application of inhibitors can also be used for other purposes. When treated with specific inhibitors blocking the competitive branch, more carbon flux will be channeled to the biosynthesis of target compounds, and result in the improved accumulation. Furthermore, if the cells are treated with specific inhibitors, the enzyme activity will be inhibited, and the upstream compounds can be consequently accumulated at higher concentration which will facilitate the identification and characterization of key intermediates.

### 3.6. Application of genetic techniques in regulating the biosynthetic pathway

With the development of molecular technology, more and more genetic information becomes available online. Until now, there are dozens of plant species whose genome has been sequenced, such like *Arabidopsis thaliana*, *Oryza sativa*, *Artemisia annua* and so on. Besides, genetic techniques (EST, RACE PCR, transformation, T-DNA tagging, siRNA, miRNA etc.) have also been carried out in other medicinal plants, like *Panax giseng*, *Saussurea involucrate* and so on. These techniques provided tons of information and possibilities for the manipulation of target compound biosynthesis. Japanese scientists introduced a flavonoid 3'5' hydroxylase gene into rose plant with red flowers, and the transgenic plant showed blue-purple flowers <sup>[12]</sup>. In *Saussurea involucrate* hairy root culture, a chalcone isomerase gene was introduced and over expressed, which resulted in 12 times production of apigenin of that in control hairy root <sup>[13]</sup>. Tyrosine decarboxylase gene isolated from parsley, was introduced in potato, and in transgenic plant, salidroside was detected <sup>[14]</sup>. Salidroside is a phenylethanoid compound with significant adaptive effects and is the main active compound in *Rhodiola sachalinensis*. This report showed us the potential of producing new pharmaceutically important compounds besides original medicinal herb.

### 3.7. Combinatorial Biosynthesis in production of useful secondary metabolites

Combinatory synthesis is a method to combine metabolism pathways of different species genetically. As the development of molecular biology, and the proposal of creative experimental methods and idea, the concept of combinatory will be changed in the future, but there is no doubt about its importance in post genomics.

Currently, most research related to combinatory synthesis are using two kind of microbes in their experiment, *Escherichia coli* and *Saccharomyces cerevisiae*. Two microbes are not accidentally selected as model species. Experimental skills related to their mutation, culture, transformation and expression are all well developed. Besides, the whole genome sequence information is also available which provided detailed information for the manipulation and genetic engineering of two microbes.

To be noticed is that, full biosynthetic pathways of secondary metabolites are not involved in the microbes or at least partially not involved. Therefore, to produce the specific compound in microbes, whole biosynthetic pathway or part of the pathway must be introduced into the target microbes (through high copy number plasmid or integration to chromosome). This is the main idea of using *E. coli* and *S. cerevisiae* as the vehicles of combinatory biosynthesis, and also the bottle-neck limiting the application of this technique, due to the poor understanding of biosynthetic pathway of secondary metabolites in plants. Thus, currently, most researchers are focusing on high-yield intermediate production and use chemical semi-synthesis to produce the final product of interest or precursor feeding in the cultured transformed microbes.

### 1. Anthocyanin production in genetic engineered *E. coli*

Due to the wide application of anthocyanin in naturally occurring dye, edible pigment, and antioxidant activity, its biosynthetic pathway has attracted attention of scientists and lots of important experimental experiences and results are reported. Its biosynthetic pathway is one of the best understood pathway of natural products. In this study, genes of flavanone 3' hydroxylase (*F3'H*), anthocyanidin synthase (*ANS*) cloned from *Malus domestica* and genes cloned from *Anthurium andraeanum*, dihydroflavonol 4-reductase (*DFR*) and UDP-glucose:flavonoid -3-O- glucosyltransferase (*3-GT*) are constructed in one artificial gene cluster and expressed in *E.coli*. In *E. coli* culture media, when fed the precursor of anthocyanin biosynthesis, the naringenin, or eriodictyol, anthocyanin production was detected in culture media <sup>[15]</sup>.

### 2. Artemisic acid production in engineered *S. cerevisiae*

Artemisine is the most effective anti-malaria compound until now, traditionally, the compound is purified from dry harvested aerial part of *Artemisia annua*. The purification is time and labor consuming, and chemical synthesis has been proved cost unattractive. Therefore, genetic engineering of yeast cell is used to produce the precursor of this compound, artemisinic acid, and semi synthesis is used to further synthesize the artemisine. Genes isolated from *A. annua*, amorphadiene synthase (*ADS*) and cytochrome P450 monooxygenase (*CYP71AV1*) were interegrated into yease chromosome. Furthermore, the biosynthesis pathway of farnesyl pyrophosphate (*FPP*) was also engineered to produce more *FPP*, and the useage of *FPP* to steroid was also blocked to increase the *FPP* flux into artemisinic acid. The engineered yeast cell was cultured in YEP media, and the yield of artemisinic acid reached 115 mg/L. besides, the purification process is also very simple that, the final product was all on the surface of yeast cells, and by centrifugation and acidic buffer washing, 95% of artemisinic acid produced can be recovered <sup>[16]</sup>.

## 4. Conclusions

Plant cell and organ culture is fast developing in the field of secondary metabolism regulation, with the development of molecular biology, deepen understanding of the biosynthetic pathways of natural products and newly developed treatment strategies. It is now not only an approach to produce target compounds, but also powerful tool for gene function research in post-genomic era. More and more medicinal herbs have been used to establish different culture systems, which will facilitate preserving the natural resources and improve the possibilities of producing compounds of interest at industrial level. In order to achieve target compounds yield high enough for industrial application, or to elucidate the full biosynthetic pathway, methods such as key gene(s) transformation, elicitor treatment, precursor feeding, inhibitor treatment should be combined in an appropriate manner. However, due to the culture characteristics and final product recovery process, combinatorial biosynthesis showed even more potential in the industrial application.

## Author details

Hu Gaosheng and Jia Jingming

School of Traditional Chinese Material Medica, Shenyang Pharmaceutical University, Shenyang, Liaoning, P. R. China

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