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Review article

Research methods to study plant secondary metabolic pathways and their applications in the analysis of the biosynthetic pathway of stilbenes from *Polygonum multiflorum* –A review

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Abstract

Plant secondary metabolites play significant roles in plant-environment interactions. Plant - derived secondary metabolites are compounds that can be widely used in industry, medicine, food sciences etc. In this review, we summarize the research methods applied to analyse biosynthetic pathways of plant secondary metabolites. Moreover we present an up to date overview of the studies that were performed on biosynthetic pathways of stilbenes. In this manuscript we put emphasis on the biosynthetic pathway of 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-D-glucoside (TSG), an active component extracted from *Polygonum multiflorum* Thunb.

Keywords: 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-D-glucoside; *Fallopia Multiflora*; isotope tracer; plant secondary metabolism engineering; *Polygonum multiflorum*; precursor feeding; secondary metabolism; stilbene.
Abbreviations: 4CL_4-coumarate: coenzyme A ligase; C4H_cinnamic acid 4-hydroxylase; CHS_chalcone synthase; PAL_phenylalanine ammonia lyase; STS_stilbene synthase; P. multiflorum_ Polygonum multiflorum; TSG_2,3,5,4'-tetrahydroxystilbene-2-O-ß-D-glucoside.

Introduction

Plant secondary metabolism has multiple functions throughout the plant's life cycle. Plant secondary metabolism utilizes compounds and enzymes from primary metabolism. Currently tens of thousands of plant secondary metabolites have been described, including terpenoids (isoprenoids), phenylpropanoids, alkaloids etc (Kennedy and Wightman, 2011; Kroymann, 2011). These plant natural products play an important role in the interaction of plants with their biotic and abiotic environment. For example, they serve as attractors for pollinators or seed dispersers. Plant-derived secondary metabolites function as defensive compounds or toxins that guard against pathogens and herbivores and protect plants from abiotic stresses such as ultraviolet (UV) light (Dixon, 2001; Verpoorte and Memelink, 2002). In addition, plant secondary metabolites have broad application potential in industry, agriculture, medicine and food,sciences (He and Giusti, 2010; Ngo et al., 2013). In the past two decades, nearly two thirds of approved new drugs were obtained from natural plant products (Newman and Cragg, 2007). Moreover, majority of the world's population relies on medicinal plants for its primary pharmaceutical care (McChesney et al., 2007). It is worth to mention artemisinin (Njuguna et al., 2012), paclitaxel (Qi et al., 2013), camptothecin (Mollica et al., 2012), vincristine (Eden et al., 2010), podophyllotoxin (Komericki et al., 2011), ginkgolide (Wang et al., 2006) as plant-borne products widely used in clinical care. The classification of plant secondary metabolites utilize following criteria. Based on chemical structure plant-derived secondary metabolites can be divided into phenolic, terpenoids and nitrogen organic material, etc. Depending on physiological activity plant secondary metabolites can be grouped into phytoalexin, growth-stimulating hormones, vitamins, pigments, alkaloids, plant toxins, etc. Finally, depending on the initial structure utilized for their biosynthesis plant-derived secondary metabolites may be divided into terpenoids, alkaloids and phenylpropanoid. *Polygonum multiflorum* Thunb (*Fallopia multiflora* Thunb.) is a perennial vine-like herb. In Chinese it is called He-shou-wu; it has been widely used in traditional Chinese medicine as a tonic and anti-aging agent for centuries. Recent studies have demonstrated that *P. multiflorum* contains many active ingredients, such as stilbenes, anthraquinones, flavonoids, phospholipids etc (Yi et al., 2007; Han et al., 2013). 2,3,5,4'-tetrahydroxystilbene-2-*O*-β-D-glucoside (TSG) is considered as a representative active ingredient of *P. multiflorum* (Hata et al., 1975; Chen et al., 2012). The Pharmacopoeia People's Republic of China used it as the quantitative index of *P. multiflorum*. Numerous studies have shown that TSG possesses extensive pharmacological activities, including anti-oxidant (Ryu et al., 2002; Valko et al., 2007), anti-inflammatory (Zhang et al., 2007; Wang et al., 2008), anti-atherosclerosis (Zhang et al., 2009; Yao et al., 2013), neuroprotective effects (Wang et al., 2007; Qin et al., 2011), etc. Therefore, it is a profound significance to elucidate the biosynthetic pathways of TSG for improving the medicinal value of *P. multiflorum* by gene regulation to produce more TSG. Plant secondary metabolites are highly diverse in structures and their metabolic pathways are complex and

varied as well. The biosynthesis and accumulation of secondary metabolites are usually organ-, tissue- and developmental stage-specific (Yang et al., 2012). However the total level of secondary metabolites is generally low in plants. Thus many plant secondary metabolites utilized as drugs are present in low amounts, facing the problems of resource shortage and high price and finally leading to limitations in the clinical healthcare. These deficiencies may be overcome by an extensive research on the biosynthetic pathways of important plant secondary metabolites, leading to the complete understanding of plant secondary metabolism. Obtained fundamental knowledge will be utilized in plant metabolic engineering technologies to produce these products in elevated quantities. Here, we provide an overview of research methods utilized to study biosynthetic pathways of plant secondary metabolites, focusing on the research progress of the biosynthetic pathways of TSG that belongs to stilbenes extracted from *P. multiflorum.*

Research methods applied to study biosynthetic pathways of plant secondary metabolites

Feeding with natural precursor compounds

The accumulation of secondary metabolites from callus and suspension cells can be enhanced by addition of precursors into the medium. When exact precursor for analyzed compound is unknown, the set of putative precursors may be applied. By comparing the content of secondary metabolites from callus and suspension cells that are fed with suspected precursors with control plants the researchers can screen for appropriate precursor of a target secondary metabolites (Exposito et al., 2009). The successful example of this approach is the enhancement of podophyllotoxin production by supplementation of cell suspension cultures of *Podophyllum hexandrum* Royle with coniferyl alcohol (biosynthetic precursor of podophyllotoxin) (Woerdenbag et al., 1990). Varying concentrations of L-phenylalanine (0, 1, 10 and 100 µM) as the putative precursor of silymarin were added to the medium of *Silybum marianum* hairy root cultures. This supplementation led to significantly enhanced accumulation of silymarin in hairy root cultures. Furthermore, the optimal concentration of phenylalanine (100 µM) and optimal feeding time (72h) for silymarin production were determined. Hence, these results strongly indicate that L- phenylalanine may be a biosynthetic precursor of silymarin (Rahimi et al., 2011). Precursors such as tyrosine, phenylalanine, caffeic acid and cucumber juice caused increased accumulation of phenylethanoid glycosides (PeGs) (by 50%, 23%, 12%, and 12%, respectively) in the *Cistanche salsa* cell suspension cultures. Moreover, under the combined feeding of precursors, the total production of PeGs in the cell suspension culture reached the highest amount of 1358.1 mgl^{-1}, which was about two-fold of that in the control. All these findings indicate that tyrosine, phenylalanine and caffeic acid may be the biosynthetic precursors of PeGs (Liu et al., 2007).

Radioactive isotope-labeled precursor's tracer

Isotope tracer technique is a classic method of exploring plant secondary metabolic pathways. Before 1960 radioactive isotope tracer was the key technology to study plant secondary metabolic pathways. At this time preparation of the stable isotopes was very expensive and of low yield and modern technologies in form of mass spectrometry (MS) and nuclear magnetic resonance (NMR) were still in their infancy. In the radioactive isotope tracer method radiolabelled

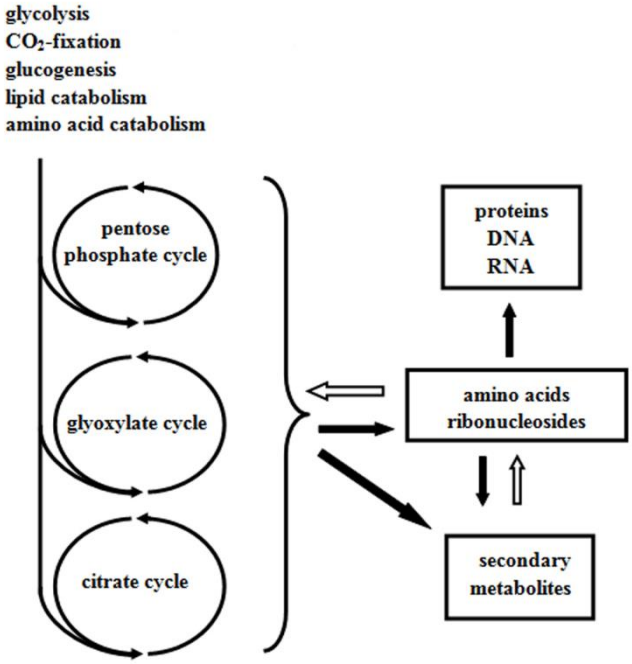

Fig 1. Biosynthesis of secondary metabolites from central metabolic intermediates and/or primary metabolites. Reconstruction of central labeling patterns and biosynthetic pathways by pattern recognition are indicated by the symbol ⇨.

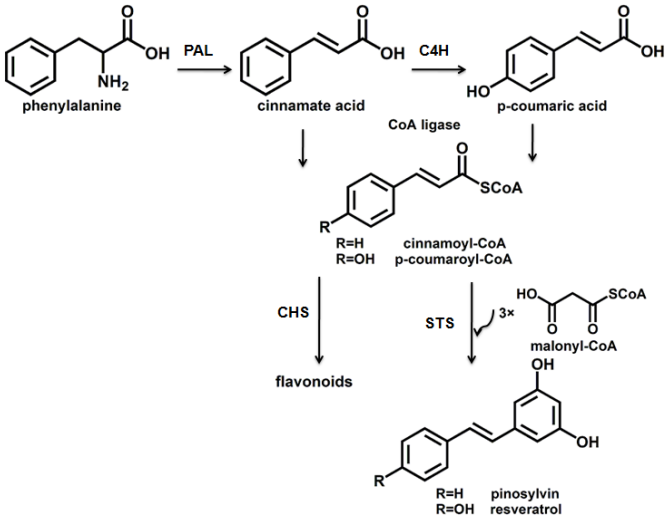

Fig 2. The biosynthesis pathway of stilbenes.

precursors are added into the whole plants, isolated organs or plant cell suspension cultures and precursor feeding is followed by isolation of target metabolite. When target metabolite contain radioactive isotope its "bona fide" precursor has been identified. Majority of the biosynthetic pathways of plant secondary metabolites such as taxol (Jennewein and Croteau, 2001), artemisinin (Akhila et al., 1987), morphine (Kirby, 1967), have been elucidated through radioactive isotope tracer technology. Taxol, clinically important secondary metabolite, was initially isolated from the bark of *Taxus Brevifolia* by Wani et al. (1971). Currently it is one of the most significant anticancer natural compounds. According to Zamir et al. (1992), the feedings of *Taxus canadensis* with radiolabelled acetate and mevalonate

resulted in formation of the radioactive taxol. These data suggest that the taxane carbon skeleton of taxol is formed via the mevalonic acid (MVA) pathway. In contrast it was proposed by *Eisenreich* et al. (1996) that the taxane carbon skeleton is derived from 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. Interestingly, the recent studies have shown that the taxane skeleton is derived from the cyclization of geranylgeranyl pyrophosphate (GGPP) (Koepp et al., 1995). The cyclized diterpenes were available from the cell suspension cultures of Pacific yew (*Taxus brevifolia* Nutt). When they were infiltrated into the *T. brevifolia* stem discs, high radioactivity taxanes such as 10-deacetyl baccatin Ⅲ and taxol were extracted (Hefner et al., 1996).

Stable isotope-labeled precursors tracer

Stable isotope tracer was an emerging technology in the 1960s. In the stable isotope-labeled precursor technique the stable isotope tracers are added to the experiment system. Stable isotope tracers will move, transform and migrate along with the same substances or participate in the metabolic activities of the system. Then the positions of the intermediates and the final products in the biosynthetic pathways can be confirmed by dynamic monitoring the metabolic changes, leading to elucidation of the biosynthetic pathways. The detection sensitivity of radioisotope tracer is much higher than that of stable isotope tracer. Moreover the measurements are less demanding, safe and analyzed system is characterized by stable physical properties. Besides all these superior properties, stable isotope tracer technology can detect several isotopes of different mass numbers simultaneously. Stable isotope tracer greatly technology contributes to the elucidation of biosynthetic pathways of secondary metabolites. Because of its superior properties, stable isotope tracer technology allowed for discovery of novel biosynthetic pathways. Moreover this technique strongly contributed to correction of inappropriately assigned metabolic pathways (Imai et al., 2006). The biosynthetic pathways of codeine and morphine were studied through stable isotope-labeled precursor tracer technology. The opium poppy (*papaver somniferum*) seedlings were supplied with ^{13}C-labeled precursors, and nanogram quantities of labeled codeine were isolated from the seedlings. The labeling degree of codeine was calculated based on LC-MS full scan data as follows: [ring-$^{13}C_6$]-L-tyrosine into codeine: 30%; [ring-$^{13}C_6$]-tyramine into codeine: 38%; [1,2-$^{13}C_2$], [6-O-methyl ^{13}C]-(R,S)-coclaurine into codeine: 57%, which indicated that the three compounds were the biosynthetic precursors of codeine(Poeaknapo et al. 2004). In order to clarify the biosynthetic pathway of 2-phenylethanol (2PE) from rose, many researchers fed rose flowers (Rosa damascena Mill., R. 'Hoh-Jun', and R. 'Yves Piaget ') with isotope-labeled precursors, and found that L-phenylalanine and phenylacetaldehyde were its biosynthetic precursors (Watanabe et al., 2002; Hayashi et al., 2004; Sakai et al., 2007). In addition, Yang et al. (2009) attempted to feed the isolated protoplasts of rose flowers with [$^2H_8$] L-phenylalanine and [2,3,4,5,6-$^{13}C_5$] shikimic acid, and finally proposed hypothetical biochemical pathway of 2PE from shikimic acid via chorismic acid, L- phenylalanine, and phenylacetaldehyde (Yang et al., 2009). *Krisa* et al. (1999) studied the ^{13}C-labeled condition of anthocyanin by adding ^{13}C-labeled phenylalanine into the *Vitis vinifera* cell suspension cultures, finding that 50-57% of labeled phenylalanine can be incorporated into anthocyanin. Thus, these results indicated that phenylalanine was the biosynthetic precursor of anthocyanin.

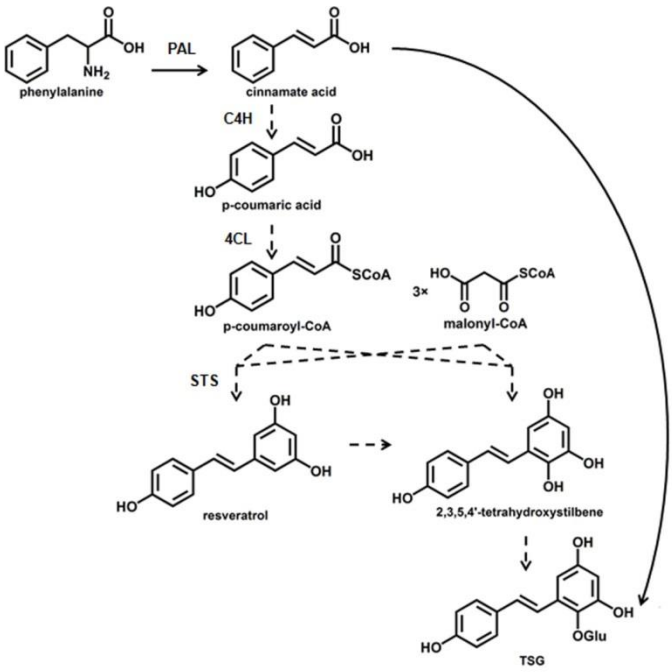

Fig 3. The biosynthetic pathway of TSG from *Polygonum multiflorum* Thunb. (The solid lines indicate that the routes have been confirmed; the dotted lines indicate hypothetical pathways)

Retrobiosynthetic nuclear magnetic resonance technology

Radioactive and Stable isotope-labeled precursors tracer technologies are applied to deduce plant metabolic pathways. This deduction implies that there is only one metabolic pathway from the precursor to the final metabolite. However, in most cases many chemicals will be able to serve as precursors in wide variety of metabolic pathways. In contrast to the utilization of specific precursors that are structurally closely related to the terminal metabolites, the retrobiosynthetic method preferentially uses general initial metabolites, such as glucose, ribose, glycerol, pyruvate or acetate (Eichinger et al., 1999; Römisch-Margl et al., 2007; Rauhut and Glawischnig, 2009). Each of these compounds can be converted to a vast variety of different metabolites. Moreover, all the biosynthetic pathways utilize initial materials that are derived from central metabolic pathways, e.g. pentose phosphate cycle, glyoxylate cycle, citrate cycle. The labeling patterns of the central intermediates, e.g. acetyl CoA, trioses, dicarboxylic acids, are usually complicated, because of the fact that central intermediates are present at low quantities and thus cannot be isolated in sufficient amounts for direct analysis. However, the labeling patterns of the complicated central metabolites can be reconstructed with high fidelity from the labeling patterns of amino acids and ribonucleotides (Werner et al., 1997; Bacher et al., 1998; Ostrozhenkova et al., 2010) (Fig. 1). Frequently, the ^{13}C NMR signals of isolated metabolites are quite complex since the isotopes from the general precursors can generally be transferred to a certain metabolite via more than one route. Furthermore, the labeled precursors can be recycled extensively by cyclic metabolic processes that cause the NMR signals of metabolites rather complex as well. Consequently, the interpretation of such intricate spectra tends to be laborious. Hence it is very important to utilize spectral simulation, deconvolution techniques and one-dimensional and two-dimensional NMR experiments to analyze these complex spectra (Eisenreich and Bacher, 2007).

Genetic engineering of plant secondary metabolism

Tracer experiments aim to elucidate the biosynthetic pathways of plant secondary metabolites. However, the isolation and identification of their metabolism-related genes cannot be studied with above-mentioned techniques. The plant genetic engineering allows for characterization of genes from biosynthetic pathway, their expression profile, analysis of the activities of encoded enzymes and their regulation. The potential achievements of genetic engineering of plant secondary metabolism will constitute theoretical foundations for directed manipulations leading to enhanced production of secondary metabolites in the future (Nascimento and Fett-Neto, 2010). There are two main approaches to modify metabolic pathways by genetic engineering: one is to import key enzyme genes of metabolic pathways, and the other is to inhibit expression of genes encoding enzymes of metabolic branches.

In this approach single or multiple key enzyme genes of target metabolic pathways are imported into the plants, and then these genes are overexpressed, leading to increased content of target secondary metabolites or synthesis of exogenous secondary metabolites. This positive regulator strategy, the most commonly used strategy of metabolic engineering nowadays, often requires a combination of multiple genes to achieve satisfactory results. For example, overexpression of a tryptophan decarboxylase (TDC) in *Catharanthus roseus* crown gall calluses results in increased tryptamine levels but not in increased terpenoid indole alkaloid (TIAs) production (Goddijn et al., 1995). However, cells of *C. roseus* were genetically engineered to over-express the enzymes strictosidine synthase (STR) and TDC, which increased the accumulation of TIAs (Canel et al., 1998). Lee et al. (2004) reported that the overexpression of *Panax ginseng* squalene synthase gene (PgSS1) in the *Panax ginseng* resulted in remarkable increase of phytosterols as well as ginsenosides. These results demonstrated that PgSS1was a key regulatory enzyme not only for biosynthesis of phytosterol but also for triterpene. A novel stilbene synthase gene from Chinese wild *Vitis pseudoreticulata* (W.T.Wang) was transferred into *V. vinifera* L. cv. Thompson Seedless via *Agrobacterium tumefaciens*-mediated transformation and the content of resveratrol was detected by HPLC. This study has demonstrated that resveratrol level in these transformants was 5.5 times of non-transformed tissues (Fan et al., 2008).

Plant primary metabolism can change into secondary metabolism and secondary metabolites have ability to transform into each other. Moreover a variety of different secondary metabolites may be synthesized by a common precursor. The interesting strategy to enhance the accumulation of "wanted" secondary metabolite is the inhibition of the activity of key enzymes of the metabolic branches ("unwanted metabolites"), thereby focusing the biosynthetic reaction towards the direction of the synthesis of the target compounds. One of the technical possibilities to affect target gene is the antisense technology (Sahu et al., 2007). In this method antisense nucleic acids are designed that are specific for the target gene sequence and based on the principles of nucleic acid hybridization they can decrease or completely shut down the target gene expression. This strategy can be used to block certain metabolic pathways or branches, thereby reducing the generation of unwanted product and enhancing the accumulation of the beneficial target product (Hebert et al., 2008). Allen et al. (2004) utilized RNA interference (RNAi) technology to silence codeinone reductase (COR) in the opium poppy (*Papaver somniferum*), which inhibits the biosynthesis of morphine. It was demonstrated that the silencing of COR has led to the decrease of morphine, codeine, oripavine and thebaine and the enhanced accumulation of the precursor alkaloid (S)-reticuline (Allen et al., 2004). In anthocyanin biosynthetic pathway, dihydroflavonol 4-reductase (DFR) converts dihydroflavonol into leucoanthocyanidin. Lin et al. (2013) cloned FaDFR gene from strawberry (*Fragaria ananassa)* fruit, and constructed its RNAi vector pBI-DFR*i*. Authors were able to demonstrate that with this specific RNAi FaDFR was downregulated, fruit color became pale and the concentration of anthocyanin decreased, indicating that FaDFR is one of the key enzymes in the anthocyanin biosynthesis pathway in strawberry fruit.

Research profile of stilbenes biosynthetic pathway in plants

The term stilbenes describes a group of plant chemicals containing the stilbene skeleton (trans-1, 2 diphenylethylene) as a basic structure (Ribeiro et al., 1999; Roupe et al., 2006). Natural stilbenes give priority to stable trans-isomer, and its main substituent is hydroxy. The number and substitution position of hydroxy group are varied. What's more, hydroxy also can be substituted by methyl, methoxyl or glucoside and the major substituted sites are 3, 4', 5 (Shen et al., 2009). Currently, stilbenes have been identified in the species of at least twenty-four families (*Leguminosae*, *Moraceae*, *Polygonaceae*, *Dipterocarpaceae*, *Vitaceae*, *Pinaceae*, etc.). Recent pharmacological studies have shown that stilbenes have a wide range of biological and pharmacological activities, including resistance to diseases, anti-oxidative, anti-aging, anti-inflammatory, cardiovascular protection etc (Rimando and Suh, 2008; Delaunois et al., 2009; Kasiotis et al., 2013). Therefore, stilbenes have important application and development potentials in plant defense reactions and human health care. In most plants, stilbenes are produced from the phenylpropanoid pathway (Fig. 2). Stilbenes biosynthesis is regulated by several enzymes, including phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4 -coumarate: coenzyme A (CoA) ligase (4CL), stilbene synthase (STS), chalcone synthase (CHS). Moreover, STS is one of the most critical regulatory enzymes (Samappito et al., 2003; Condori et al., 2009; Kiselev et al., 2009). In the phenylalanine metabolic pathway, phenylalanine, as the initiating molecule is converted into trans-cinnamic acid under the catalysis of PAL. Then, C4H transforms the cinnamic acid into p-coumaric acid (4-coumaric acid). By the action of 4CL, p-coumaric acid is transformed into 4-coumaroyl-CoA (Yu and Jez, 2008). According to the different substrates, STS can be divided into two categories: one called Akamatsu synthase (found in pinus; catalyzing the formation of Akamatsu hormone (3, 5-dihydroxy-stilbene) from malonyl-CoA and cinnamoyl-CoA) and another called resveratrol synthase (also known as 3, 4', 5 - trihydroxy stilbene synthase; found in grapes, peanuts, knotweed and other angiosperms; catalyzing formation of resveratrol (trans 3, 4', 5 – trihydroxystibene) from malonyl-CoA and coumaroyl-CoA, to form) (Preisig-Muller et al., 1999). In addition, by the catalytic action of CHS, coumaroyl-CoA can also enter the biosythesis branches of flavonoids and isoflavones (Winkel-Shirley, 2001; Winkel-Shirley, 2002). At present, it is generally believed that the biosynthesis of stilbene compounds refer to the biosynthesis of resveratrol. By the action of STS, one unit of coumaroyl-CoA and three units of malonyl-CoA are condensed into resveratrol. Next, resveratrol is further converted into different structures of stilbene compounds through hydroxylation, glycosylation etc. Furthermore, STS possesses

broad substrate specificity. This feature can be used to produce novel compounds, as minor modifications of the substrates can be used to direct the enzyme reaction to form a variety of diverse and novel products (Morita et al., 2001). STS, a member of the type III polyketide synthases (PKSs) superfamily, exists in plants in the form of gene families (Watanabe et al., 2007). STS has extensive homology to CHS which is responsible for the generation of chalcones in many higher plants (Vannozzi et al., 2012). CHS is ubiquitous in plants, but STS is only found in species that accumulate resveratrol or related compounds. Therefore, STS is essential for the bioengineering of stilbenes, like resveratrol.

Research progress of stilbenes biosynthetic pathway in *Polygonum multiflorum*

Polygonum multiflorum is a traditional Chinese herb and has been used in China for thousands of years. 2,3,5,4'-tetrahydroxystilbene-2-O-ß-D-glucoside (TSG) is one of the main active ingredients extracted from the root of P. multiflorum (Hata et al., 1975; Chen et al., 2012). Current TSG studies mainly focus on its separation, extraction, detection and on analysis of its pharmacological activities. In contrast, studies of its biosynthetic pathway are scarce. TSG and resveratrol belong to stilbene compounds and currently the studies on the stilbenes biosynthetic pathway concentrate on the trihydroxystibenes resveratrol and its glucoside derivatives which are catalyzed by STS (Yu and Jez, 2008). Interestingly, *P. multiflorum* produce the tetrahydroxys-tilbenes such as TSG, which biogenesis pathway is currently unknown. There are three possibile biosynthetic routes of TSG. TSG may be synthesized by the hydroxylation reaction of resveratrol. In the second possible pathway putative STS may catalyze formation of the coumaroyl-CoA which is then modified through hydroxylation and malonyl-CoA to form the tetrahydroxystilbene TSG. In this pathway the intermediate step in the form of resveratol is omitted. In the third hypothetical pathway, *P. multiflorum* doesn't contain STS and TSG may be synthesized by the catalytic reaction of another enzyme whose functions may be similar to STS.

Our reaserch is focused on the biosynthetic pathway of TSG from *P. multiflorum*. We utilize stable isotope-labeled precursor tracer technology to study its biosythesis. Suspension cells are the first step in the establishment of plant secondary metabolite biosynthesis. We were able to induce the calli of *P. multiflorum* and to further cultivate its friable calli thus establishing the cell suspension cultures of *P. multiflorum*. Next, the cell suspension cultures with high quantities of TSG were obtained by optimization of the culture conditions (Shao et al., 2012). Some putative precursors, like L-phenylalanine, cinnamic acid, sodium acetate and tyrosine, were screened in the cell suspension cultures. These studies have demonstrated that phenylalanine, cinnamic acid and sodium acetate may represent precursors of TSG. These initial results were confirmed by the supplementation of suspension cells with (U-$^{13}C_9$) phenylalanine and (U-$^{13}C_9$) cinnamic acid, which resulted in the incorporation of radioactivity into TSG. These data suggest following biosynthetic pathway of TSG: a precursor in form of phenylalanine is converted into cinnamic acid by PAL (Shao et al., 2010). Next, TSG is synthesized in additional enzymatic steps (Fig. 3). The utilization of genetic engineering of plant secondary metabolism allowed us to study the gene expression and functional analysis of TSG metabolism-related enzymes. Several methods were applied to extract the RNA of *P. multiflorum* and finally the protocol that allows for isolation of high-quality and intact RNA from the tuberous roots of *P. multiflorum* was established (Chen et al., 2012). A type III polyketide synthase FmPKS (FmSTS) cDNA and its corresponding gene (GenBank accession No. 411432) were isolated from the rhizomes of *P. multiflorum*. The full-length FmSTS cDNA is 1,228 bp and contains an open reading frame of 1,137 bp encoding a 378 amino acid protein. The FmSTS gene is interrupted by three introns. Southern blotting analysis have demonstrated that there are three to four copies of the FmSTS gene in the genome, grouping FmSTS to the multigene family (Sheng et al., 2010). The FmSTS was expressed in *E. coli* and the purified recombinant protein can catalyze formation of resveratrol *in vitro*, utilizing malonyl CoA and coumaroyl-CoA as substrates. The overexpression studies further demonstrated that FmSTS is a key enzyme of the biosynthesis of TSG (Zhu and Zhao, 2012). Interestingly, additional type III polyketide synthase FmPKS2 was isolated from the rhizomes of *P. multiflorum*, which shares similarities with chalcone synthases (CHSs) of *Polygonaceae* family. FmPKS2 was initially speculated to be the CHS of *P. multiflorum* (Sheng and Zhao, 2010). Clearly, further experiments are required to determine the function of FmPKS2 gene.

Conclusion

Plant secondary metabolites have multiple functions throughout the plants life cycle. Interestingly, plant secondary metabolites have a great potential for industry, agriculture, medicine, and food sciences. Therefore, the understanding of their biosynthetic pathways is of a great significance for fundamental and applied sciences. With the development of science and technology the research methods to study biosynthetic pathways of plant secondary metabolites clearly evolved, but there are still some pitfalls. Radioactive isotope-labeled precursor tracer technology has the advantage of high sensitivity and convenient detection, but it produces radioactive contaminations. Although stable isotope-labeled precursor tracer technology doesn't generate the radioactive contamination, it has the disadvantage of lower sensitivity and high cost. Retrobiosynthetic nuclear magnetic resonance technology has the disadvantages of complicated and time consuming data analysis processes. Plant secondary metabolism genetic engineering has developed rapidly in recent years, demonstrating the broad application possibilities. However, the diversity of plant secondary metabolites coupled with the complexity of related enzymes and the regulation of gene expression increase the difficulty of the secondary metabolic engineering. Moreover, the overall regulation of plant secondary metabolism and the coordination between secondary metabolic pathways are still poorly understood. The biosynthetic pathways of stilbenes are relatively simple. The studies on TSG from *P. multiflorum* have led to progress in the field; however further work is clearly required. The future identification of unknown intermediates of TSG should allow for completion of TSG biosynthetic pathway. This goal can be achieved by utilization of already identified, radiolabelled precursors, like phenylalanine. Family of stilbene synthases in *P. multiflorum* contains at least three members. Only one of FmSTSs had been analyzed in detail so far. Additional experiments are urgently required to clarify the biological function of other family members of FmSTS. In the phenylpropanoid pathway STS competes with CHS for the same substrates. It is tempting to speculate that the inhibition of the biosynthetic pathway of chalcones may lead to enhanced production of stilbenes. In future, all of the secondary metabolism-related enzymes will be characterized through the transcriptome sequencing, which will lay a solid foundation for the metabolic engineering of TSG from *P. multiflorum.*

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