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Relevant Enzymes, Genes and Regulation Mechanisms in Biosynthesis Pathway of Stilbenes

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ABSTRACT

Stilbenes are natural phenolic compounds which function as antimicrobial phytoalexins in plants and affect human health as cardioprotective, antibaceteria, antioxidative and antineoplastic agents. In this review, the progresses of study on relevant enzymes, genes, and regulation mechanism in biosynthesis pathway of stilbenes are described. Here we introduce a holistic and systematic method of researching relevant enzymes, genes and other regulatory factors in bio-synthesis pathway of stilbenes—Systems biology. The application of knowledge of relative enzymes, genes and regulation mechanisms in stilbenes biosynthesis in metabolic engineering which is used as a tool of improving the disease resistance of plants and health caring quality of crops is also discussed.

Keywords: Stilbenes; Relevant Enzyme; Regulation Mechanism; Biosynthesis Pathway; Systems Biology

1. Introduction

Stilbenes, a kind of phytoalexins, are low-molecular-weight defensive substances produced by plants in response to infection after exposure to microorganisms. They are widely distributed in the plant kingdom, being reported from bryophytes and pteridophytes through gymnosperms and angiosperms. Stilbenes display a wide range of biological activitivies, such as antibacterial, antifungal, estrogenic, antitumoral [1], cardioprotective [2] and tyrosinase inhibitory activity [3]. There is great interest in their potential health benefits and capacity to improve the disease resistance of plants [4]. Much effort has been directed at the Stilbenes' extraction, structure determination, biological activity over the past decades. In recent years, some success also has been achieved in the metabolic regulation and gene engineering of stilbenes. However, their detailed biosynthesis pathways and metabolic regulation, especially complicated regulation mechanism and expressing of genes and enzymes are unknown. So it is significant to shift focus from previous research priorities to search relevant enzymes, genes, abiotic stress and biotic signals so as to elucidate their detailed biosynthesis pathway and understand metabolic regulation networks. The elucidating of stilbenes' biosynthesis pathway and regulation mechanism is believed to contribute to improve the disease resistance of plants and health caring quality of crops and also provide an opportunity to know more about global regulation networks and coordination between each pathway of secondary metabolism.

2. The Relevant Enzymes and Regulation in Biosynthesis Pathway of Stilbenes

2.1. The Relevant Enzymes and Regulation in Phenylpropanoid Pathway

The phenylpropanoid pathway is one of the most important plant secondary metabolism pathways and it is involved in the synthesis of a wide variety of important natural products from plants including flavonoids, lignins, coumarins, and stilbenes [5]. Phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumarate: CoA ligase (4CL) are key enzymes in this pathway [6]. PAL, the first and key enzyme of the phenylpropanoid sequence, is the bridge between primary metabolism and secondary metabolism. PAL catalyzes the formation of transcinnamicacid by nonoxidative deamination of L-phenylalanine, which could be the rate-limiting step in the phenylalanine metabolism pathway. It produces precursors for a variety of secondary metabolites such as flavonoids, lignins, coumarins, and stilbenes. PAL genes are transcriptionally activated after microbial infection or treatment of plant cells with microbial elicitors [7]. The second step in the phenylpropanoid pathway is the hydroxylation of trans cinnamic acid to 4-coumaric acid, which is catalyzed by C4H, a cytochrome P450 monooxygenase [8,9]. C4H is induced by light, elicitors,

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and wounding [8,10-12]. Its induction often is closely coordinated with PAL induction [13]. The 4-coumaric acid is then activated to its CoA thioester by 4CL. 4-coumaroyl CoA is funneled into branched pathways leading to a wide array of phenolic metabolites, including lignin, flavonoids [5].

Phenylpropanoid biosynthesis comprises reactions through which metabolic channeling may occur. Metabolic channeling offers unique opportunities for enhancing and regulating cellular biochemistry and major advantage of such spatial organization is the transfer of biosynthetic intermediates between catalytic sites without diffusion into the bulk phase of the cell [14]. This phenomenon involves the physical organization of successive pathway enzymes into complexes through which metabolic intermediates are channeled [15].

Studies demonstrate phenylpropanoid pathway and flavonoid metabolism branch are assembled as a linear array of sequential enzymes loosely anchored to the cytoplasmic face of endoplasmic reticulum membranes [7,16-17]. For example, Cytochrome P450 enzymes, such as C4H, Flavanone-3-hydroxide transketolase, the ferulic acyl-5-hydroxylation enzyme are anchored to the external surface of the endoplasmic reticulum [18-20]. PAL and C4H activities are colocalized on membranes of the endoplasmic reticulum. This organization regulate the partitioning of intermediates among competing pathways and determine the intracellular deposition of end products. PAL, CHS, STS, isoflavonids synthase are structure specific enzymes, Flavanone-7-O-methyltransferase, isoflavones-4-O-methyltransferase and isoflavones (isoflavanone) dimethylallyltransferase are modification enzymes in the phenylalanine metabolism pathway.

2.2. The Relevant Enzymes and Regulation in Biosynthesis Pathway of Stilbenes

Stilbene phytoalexin is derived from phenylalanine via the general phenylpropanoid pathway [21]. The last step is catalysed by Stilbene synthase (STS) which is the key enzyme of the biosynthesis pathway. STS provides the first committed step by catalyzing the sequential decarboxylative addition of three acetate units from malonyl-CoA to a p-coumaroyl-CoA starter molecule derived from phenylalanine via the general phenylpropanoid pathway (**Figure 1**). For example, Resveratrol synthase (STS, EC 2.3.1.95) condenses three molecules of malonyl-CoA and one molecule of cumaryl-CoA to form resveratrol. In the same active site, chalcone synthase (CHS) can catalyse the formation of chalcone by p-coumaroyl-CoA and malonyl-CoA via the intramolecular cyclization and aromatization of the resulting linear phenylpropanoid tetraketide [22]. Downstream modification enzymes in branching pathways produce a number of biologically important compounds.

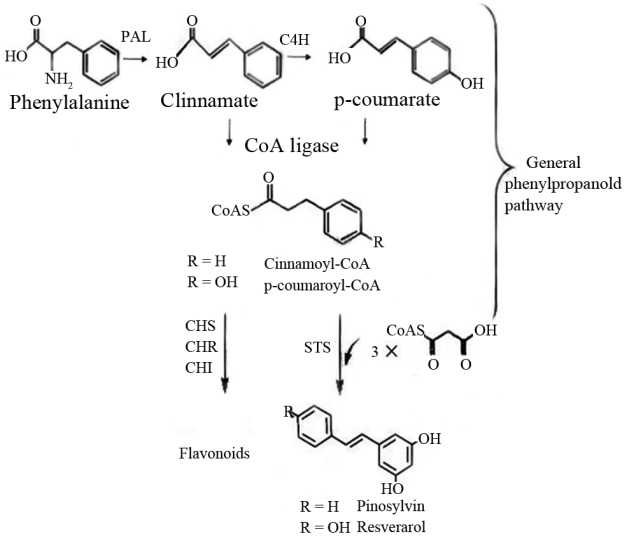

Figure 1. The biosynthesis pathway of stilbenes.

Different kinds of enzymes are involved in the biosynthesis pathway of stilbenes, which include highly species- and substrate- as well as stereospecific enzymes, modifycation enzymes and others act as regulators. Some well studied enzymes, PAL, 4CL, C4H, Pinosylvin methoxy transferase (PMT) in combination with STS are responsible for the regulation of biosynthesis of stilbenes. There are also other enzymes which are involved in the in the biosynthesis pathway and metabolic regulation of stilbenes are not definite. However, all the enzymes in the biosynthesis pathway of stilbenes can be classified according to their substrate specificity: stereochemical specific enzymes catalyse the formation of backbone of stilbenes and enzymes catalyse the modification reaction of the products which the first kind of enzymes synthesize. The first kind of enzymes include PAL, CHS, STS of phenylpropanoid pathway. The stilbene backbone is synthesized from cinnamoyl-CoA and malonyl-CoA by STS. The study on the capacity for building novel and unusual polyketides from alternative substrates of STS has shown that minor modifications can be used to direct the enzyme reaction to form a variety of different and new products [23]. The second kind of enzymes include hydroxylation enzyme, dehydrogenase, oxidase and glycosyltransferase. The specificity of these enzymes on its substrate group permutation is not high, this may be one of the main reasons why plant tissues can synthesized a series of relevant secondary metabolites [24].

The activity of key enzymes is not the only factor that affects the accumulation of stilbenes in the plants, all the enzymes involved in the formation and regulation of stilbenes as well as the supply of substrates are also associated with the accumulation of end product. The regulation mechanism of metabolic channeling also play an important role in the induction and accumulation of end

product in the branch pathway of stilbenes. The activating of metabolic channeling and biosynthesis pathways of homologous synthesis is determined by the expression of the key enzymes, but the accumulation is most determined by the expression of rate-limiting enzymes in plants. Rate-limiting enzymes which usually can be found at the branch point or the downstream of biosynthesis pathways of secondary metabolites in plants are responsible for the synthesis of precursors of many secondary metabolites [25]. Interactions between transcription factors and coordinate expression of different enzymes in the metabolic channeling have synergistic effects on the accumulation of the stilbenes. Relevant enzymes in metabolic channeling can form multienzyme complexes and coordinated express in different parts of cell. Enhanced coordinate expression of the enzyme complex can lead to a dramatically accumulation of end products. For example, induced coordinate expression of PAL and STS in the biosynthesis pathways of stilbenes can affect the synthesis of the stilbenes [26].

The biosynthesis pathway of the stilbenes in plants is closed in general conditions, which is only activated in response to microbial infections and other environmental inducers. Environmental factors including biotic and abiotic stimuli, carbon-nutrition balance, genotype and ontogenesis usually control and regulate the biosynthesis of secondary metabolites in plants [27-29]. The enzymes involved in the biosynthesis pathway of stilbenes express after the expression of corresponding genes, and the expression level of the enzyme genes is under strict regulation in plant cells due to coordinate control of the biosynthetic genes by transcription factors [30]. Coordinate transcriptional control of biosynthetic genes emerges as a major mechanism dictating the final levels of secondary metabolites in plant cells [31]. This regulation of biosynthesis pathways is achieved by specific transcription factors encoded by genes unlinked to the biosynthetic gene clusters which regulate multiple physiological processes and generally respond to environmental cues such as pH, temperature, nutrition. Transcription factor activety itself is regulated by internal signals, for example plant hormones, or external signals such as microbial elicitors or UV light. Stress hormones, such as ethylene, jasmonic acid, and salicylic acid, induce STS mRNA accumulation in leaves of mature peanut plants. The expression of resveratrol synthase (RS) genes is induced by biotic and abiotic factors in peanut cell cultures [32]. Formation of pinosylvin (PS) and pinosylvin 3-O-monomethyl ether (PSM), as well as the activities of STS and S-adenosyl-l-methionine (SAM): pinosylvin O-methyltransferase (PMT), were induced strongly in needles of Scots pine seedlings upon ozone treatment, as well as in cell suspension cultures of Scots pine upon fungal elicitation [33]. A modeling method for the induction of resveratrol synthesis by UV irradiation pulses in Napoleon table grapes is proposed. Cantos etc. use the controlled UV irradiation pulses as a simple postharvest treatment to obtain possible "functional" grapes with enhanced health-promoting properties high resveratrol content [34].

3. The Application of Systems Biology in the Research on Relevant Enzymes and Genes in Biosynthesis Pathway of Stilbenes

Systems biology is a new science which makes us be able to understand biological systems grounded in the molecular level as a consistent framework of knowledge for the first time after the genomics, proteomics etc. were put forward [35]. It is such a rapidly evolving discipline endeavours to study the detailed coordinated workings of entire organisms with the ultimate goal to understand the dynamic networks of regulation and interactions that allows cells and organisms to live in a highly interactive environment [36]. The well studied molecular biology only care about individual gene and protein. However, systems biology is the study of cell signaling and gene regulatory networks and components and functions of the biological system, can also be understood as the study of all components in a biological system (genes, mRNA, protein etc.) and the interactions between these components in a certain circumstances [37]. Systems biology is a powerful tool to comprehensively explore the biological system, the application of it's thinking model in secondary metabolites of medicinal plants brings us into a new era of understanding how to connect genes to metabolites by a systems biology approach [38].

3.1. Systems Biology Research Methods

The classical molecular biology research is to search for specific genes at the DNA level, and then to study gene functions by gene mutation, gene knockout and other means, it also can be described to study individual genes and proteins by using a variety of means. Genomics, proteomics, transcriptomics etc. are used as single means to research multiple genes or proteins at the same time. But using one of them alone provides only part of information of system without any details of interactions between components of system. Systems biology integrate genomics, proteomics etc. and molecular biology in order to provide complementary datas. It is enabled by recent advances in multidisciplinary scientific disciplines and high-throughput approaches that allow for the parallel large-scale measurement of biomolecules, such as mRNA, proteins and metabolites [39].

Functional genomics with the goal of characterization functions of genes has become an important method of systems biology. It provides comprehensive analysis of gene functions at the genome or system level, which shift

focus from research of single gene or protein to multiple genes or proteins using high-throughput experimental methods in combination with mass datas of statistical calculation method. The technology of T-DNA insertion, transposon technology, classic subtraction hybridization, differential screening, cDNA difference analysis, mRNA differential display of gene, and serial analysis of gene expression (SAGE) of systematic analysis, cDNA microarrays and DNA chip are used to analyse information of genome sequence and elucidate the gene functions. Functional genomics is a powerful tool to reveal the biosynthesis pathways of secondary metabolites, and it will provide a solid theoretical basis for the production of secondary metabolites using metabolic engineering of medicinal plants, as well as the cell or tissue culture combined with metabolic engineering [40].

The methods of transcriptomics include differential display, gene chip, expressed sequence tags (EST) analysis, massively parallel signature sequencing (MPSS), amplified fragment length polymorphism (AFLP) [41,42]. Two-dimensional electrophoresis, mass spectrometry technology bioinformatics analysis are primary methods of screening and identifying proteomics. Yeast two—hybrid system (Y2H), tandem affinity purification (TAP) can be used to study protein—protein interactions and green fluorescent protein (GFP) as maker to study subcellular localization. Metabonomics is a very important tool to study medicinal plants and promote modernization of traditional Chinese medicine [43,44], includeing nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), combined application of fourier transformation mass spectrometry (FTMS) and capillary electrophoresis-mass spectrometry (CE-MS) [45].

Technologies of Genomics, proteomics, transcriptomics and metabolomics detect the various molecules and study their functions at DNA, mRNA, protein and metabolite levels.

3.2. The Application of Systems Biology Approaches in Research on Related Enzymes and Genes in Biosynthesis Pathway of Stilbenes

When we study stilbenes with thinking model of systems biology, various levels of information including the DNA, mRNA, small molecules, proteins and protein interaction networks should to be integrated in order to obtain a series of relevent enzymes, genes or regulatory factors in it's biosynthesis pathway. All the information can be used to construct a reasonable model in order to elucidate the biosynthesis pathway, regulation mechanisms of stilbenes [46]. For example, the study of relative enzymes and genes in biosynthetic pathway of tanshinone using thinking model of systems biology. Groups of materials with phenotypic differences are analysed in order to get datas of metabolomics, proteomics, transcriptomics which can be gained with gene chips. Systemic results about genes and enzymes revolved in the biosynthesis pathway of tanshinone were obtained [47-50]. As a result, an unique new branch of two terpene biosynthesis of tanshinone biosynthesis was found.

Our group focused the research on biosynthesis pathway of a kind of stilbenes, stilbene glucoside (2,3,5, 4'-tetra-hydroxy-stilbene-2-O-*β*-D-glucoside) which is the major bioactive principles in Polygonum multiflorum (**Figure 2**). The application of Systems biology in the research on relevant genes and enzymes in biosynthesis pathway of stilbene glucoside can be described as follows. Firstly, the possible relevant genes or enzymes should be identified from Polygonum multiflorum. The clone of a type III polyketide synthase gene (FmPKS) using oligonucleotide primers designed for regions conserved amongst STS (with special attention given to the closely-related species of the Polygonaceae family) in Fallopia multiflora have be conducted by Shujing Sheng [51]. The FmPKS has been identified strongly correlates with the accumulation of stilbene glucoside by using Northern blotting, RNA inference and over expression, suggesting that FmPKS might play an important role in its biosynthesis. Resveratrol (**Figure 1**), another kind of stilbenes, is similar to stilbene glucoside in structure. Zhongyu Liu has investigated the overexpression of a resveratrol synthase gene (PcRS) from Polygonum cuspidatum which is closely related to the Fallopia multiflora in transgenic Arabidopsis. As a result, it causes the accumulation of trans-piceid with antifungal activity [52]. The foregoing research may be summed up to search for individual possible genes or enzymes involved in biosynthesis pathway of stilbene glucoside and study gene functions using the classical molecular biology.

Secondly, search for the possible relevent genes, enzymes through approaches of functional genomics, transcriptomics, proteomics. At present, suppression subtracttive hybridization (SSH) was performed to search for genomic differences. A subtractive cDNA library was constructed by using cDNA from Polygonum multiforum root tubers with high content of stilbene glucoside as tester and low content as driver for the subtractive hybridization. As a result, 11 clones were obtained as the differentially expressed candidates which play an important roles in further validation of genes involved in the

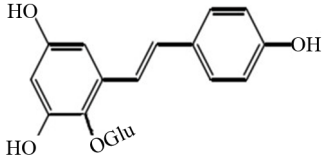

Figure 2. 2,3,5,4'-tetra-hydroxy-stilbene-2-O-*β*-D-glucoside.

biosynthesis pathway of stilbene glucoside in Polygonum multiflorum. Rapid-amplification of cDNA ends (RACE) was carried out to gain the full-length cDNAs. The application of RNA interference and over expression of these genes will reveal the specific functions of these genes and screen the genes involved in the biosynthesis pathway of stilbenes.

Thirdly, internal components (such as genetic mutation, interference) or external conditions of the Polygonum multiflorum is to be changed. The accumulation of stilbene glucoside, gene expression and enzyme expression in these cases should be detected. Next all the relevant information should be integrated to derivate how they regulate the biosynthesis of stilbene glucoside. The second step and third step ought to be repeated to revise and refine the model through a large number of experimental results, in order to finally gain all the relevent genes, enzymes and regulatory factors in the biosynthesis pathway of stilbene glucoside. These researches are from the thinking model of systems biology at different levels, which has important significance in revealing the biosynthesis pathway and regulation mechanism of stilbene glucoside in Polygonum multiflorum. The discussion and related diagrams give a good idea of the application of systems biology in research on relevent genes and enzymes in biosynthesis pathway of stilbene glucoside (**Figure 3**).

4. The Application of the Research on Enzymes and Genes in Biosynthesis Pathway of Stilbenes

The elucidating of stilbenes' biosynthesis pathway and regulation mechanism can accelerate the course of metabolic engineering as a tool for plant disease control and hunman health promotion. The increasing maturity of the plant genetic engineering technology promote the research on the biosynthesis pathway of stilbenes, and the two well studied field are stilbene synthase gene engineering and its transcription factors or regulation engineering.

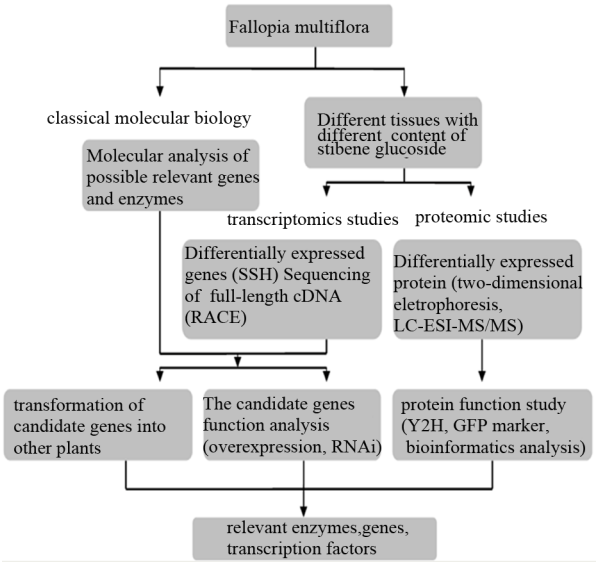

Figure 3. The application of systems biology in research on relevent genes and enzymes in biosynthesis pathway of stilbene glucoside.

4.1. The Genetic Engineering Research of Metabolic Key Enzymesin Biosynthesis Pathway of Stilbenes

STS plays an important regulatory role in biosynthesis pathway of resveratrol and other stilbene phytoalexin. An important goal of STS gene engineering is the genetic improvement of plants in increasing resistance against diseases. Namely transformate target plant genomes with chimeric strong promoter and STS gene through transgenic technology, which makes transgenic plants express STS in order to start the stilbene biosynthesis pathway, and change the plant traits and enhance plant defense against external violation [53].

Grape STS gene was introduced into *Nicotiana tabacum*, which results in transgenic tabacum express the STS and synthesize resveratrol and then dramatically increase its resistance against diseases [54,55]. The transformation of rice, wheat and other crops with grape STS gene also increase the resistance level against disease significantly of transgenic crops [56]. At present, the transgenic plants with STS gene include apple, poplar, papaya, white poplar, rape, wheat, peas, tomatoes, lettuce, hops, Arabidopsis thaliana [57-67]. Through introducing STS gene, most of transgenic plants can exhibit STS activity and synthesize exogenous stilbene phytoalexin.

4.2. The Gene Engineering of Regulating Gene or Transcription Factor in Biosynthesis Pathway of Stilbenes

Cotransformation of two or several related enzyme genes in downstream of biosynthesis pathway can be used when study stilbenes' biosynthesis involves multiple genes expression. In this way, the new secondary biosynthesis branch can be introduced into the plant then to increase the content of stilbene secondary metabolites in the transgenic plant or synthesize exogenous stilbenes. Coordinated expression of enzyme genes in secondary metabolism channel and the same or similar cisacting elements of regulatory sequences of these genes are regulated by the same transcription factors or regulation genes. Therefore, enhancing the expression of transcription factor genes and regulation genes of these important enzyme genes is a feasible way to achieve enhanced coordinated expression of multiple genes. This requires more under-

standing about identification and regulation of enzymes gene involved in biosynthesis pathway of stilbenes.

5. Summary

Now we have only a rudimentary grasp of the basic framework of the main plant secondary metabolic pathways such as alkaloids synthesis pathway, phenylpropanoid biosynthesis pathway and isoprenoid biosynthesis pathway, but lack of research on rate-limiting steps, related isozymes and specific biosynthesis pathways of specific secondary metabolite. Plant secondary metabolism is a complicated dynamic process which is regulated by plant genetic background and growth process, also affected by ecological environment and pathogen infection, insect feeding and stimulation of various elicitor. The induction expression of related genes and enzymes also need be further studied. Because of disease resistance and medicinal health functions of resveratrol, stilbene glucoside and other inducible stilbenes, secondary metabolic engineering can be used to increase the content of exogenous stilbenes in transgenic plants. However, the complexity of enzymes in biosynthesis pathway and regulation of gene expression increase the difficulty in researching metabolism genetic engineering of stilbenes. The thinking model of systems biology can be used to research the biosynthesis pathway of secondary metabolites and understand sequence of events of intermediate products and final products. The enzymes and their genes expression and regulation in each reaction step as well as the interaction of each biosynthesis branch can be definited from the view of metabolic channeling. Then after continuous integration and analysis, the detailed metabolic pathways and regulation mechanism of stilbenes will be finally elucidated. This will be the future research emphasis and direction for people to understand about regulations of secondary metabolic pathways in plants and coordination between the secondary metabolic pathways.

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