Appl Microbiol Biotechnol (2016) 100:7407–7421
DOI 10.1007/s00253-016-7723-1

MINI-REVIEW

Strategies for enhancing resveratrol production and the expression of pathway enzymes

Yao Lu[1] · **Dongyan Shao**[1] · **Junling Shi**[1] · **Qingsheng Huang**[1] · **Hui Yang**[1] · **Mingliang Jin**[1]

Received: 11 May 2016 / Revised: 29 June 2016 / Accepted: 1 July 2016 / Published online: 12 July 2016

Abstract *Trans*-resveratrol (trans-3,5,4′-trihydroxystilbene) is one of the most promising stilbenes, a type of natural phenol that is produced naturally by some plant species in response to stress. Resveratrol exhibits multiple bioactivities and is used in the agriculture, medical, food, and cosmetic industries due to its antitumor, anti-inflammatory, cardioprotective, and antioxidant properties. Due to the increasing demand, an active area of investigation is the use of plant cell culture and metabolic engineering techniques to produce large quantities of active resveratrol. However, most recent studies have focused on the efficiency of synthesizing resveratrol in vitro, but have not investigated the contributions of the transcriptional activities of the genes encoding the related enzymes in the biosynthesis pathway. This article reviews recently developed methods for the biosynthesis of resveratrol and comprehensively reviews the current state of knowledge of the function of the key pathway enzymes in resveratrol synthesis. Approaches for enhancing resveratrol production, such as introducing non-pathway genes and co-localizing enzymes are described in detail.

Keywords Resveratrol · Biosynthesis pathway · Phenylpropanoid pathway · Metabolic engineering · Key enzymes

✉ Junling Shi
sjlshi2004@nwpu.edu.cn

[1] Key Laboratory for Space Bioscience and Biotechnology, School of Life Sciences, Northwestern Polytechnical University, 127 Youyi West Road, Xi'an, Shaanxi Province 710072, China

Introduction

Resveratrol (trans-3,5,4′-trihydroxystilbene) is a phenolic compound that belongs to the stilbene class and was first isolated from white hellebore in 1940 (Takaoka 1940). It is widely found in diverse plants, including Japanese knotweed, peanuts, grapes, blueberries, cranberries, and even ferns (Jeandet et al. 2012; Li et al. 2015a; Lim et al. 2011). In nature, resveratrol exists in two geometric isoforms, *trans*- and *cis*-resveratrol (Fig. 1). The *trans*-isomer is more stable than the *cis*-isomer due to the steric hindrance between aromatic rings but can be transformed to *cis* by UV treatment (Hasan et al. 2012). *Trans*-resveratrol is the major product of stilbene synthase (*STS*), and the latter is an important target enzyme that is widely used in metabolic engineering and plant cell cultures (Chong et al. 2009; Hasan et al. 2012). In addition, glycosylation is a common modification of resveratrol that is catalyzed by glucosyltransferases and generates two corresponding piceid, *trans*- and *cis*-piceid (Fig. 1). This modification allows subsequent storage in vacuoles and may both protect plant cells from potentially toxic effects caused by resveratrol and protect the resveratrol from oxidation and enzymatic degradation (Giovinazzo et al. 2012).

Resveratrol is a phytoalexin in plants and is synthesized by phenylpropanoid pathway in response to biotic and abiotic stresses including pathogen infection, UV radiation, and mechanical wounding. In the resveratrol biosynthesis pathway in plants, resveratrol is bioconversed from one p-coumaroyl-CoA and three malonyl CoA by stilbene synthase (*STS*). Over-expression of *STS* gene in transgenic plants is an efficient way to increase their resveratrol content as well as their antioxidative properties and resistance to pathogens (Delaunois et al. 2009). Dabauza et al. (2015) over-expressed stilbene synthase gene (*Vst1*) in grapevine (*Vitis vinifera* L.) and found that the concentration of resveratrol in

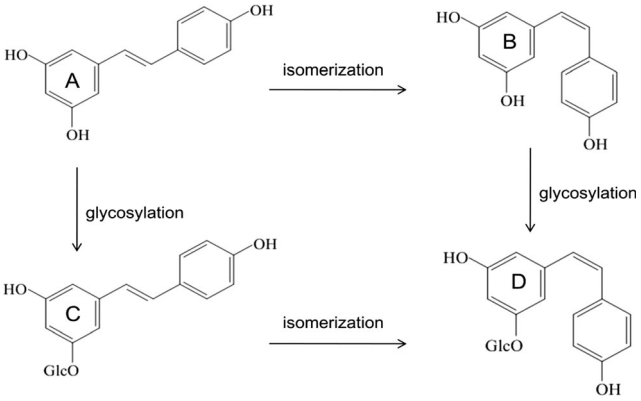

Fig. 1 Chemical structures of resveratrol and piceid (polydatin). *A* and *B*, *trans*- and *cis*-resveratrol; *C* and *D*, *trans*- and *cis*-piceid. *GlcO*, β-D-glucosyl ($C_6H_{11}O_5$)

the genetically modified plant was up to 7.5-fold higher compared to the control and resulted in increased resistance to *Botrytis cinerea*. Resveratrol has received widespread attention for its promising pharmaceutical and nutritional values (Belchi-Navarro et al. 2012). Over the last 50 years, numerous studies have found that resveratrol can provide anticancer, cardioprotective, antioxidant, and neuroprotective activities for human health and may also exert lifespan-extending properties (Shi et al. 2014). Resveratrol has been found effective in the inhibition of various tumor cells (Jeandet et al. 2012). It exhibits antitumor activity by altering gene expression by controlling the expression of a small RNA and through the regulation of a series of transcription factors (Mei et al. 2015). Resveratrol also alters many cellular processes, including cell apoptosis, cell proliferation, and cell cycle progression (Leifer and Barberio 2016). Some studies have reported resveratrol has an anti-aging effect on mice, yeast, nematodes, and fruit flies (Lancon et al. 2012; Whitlock and Baek 2012). The mechanism of this effect is thought to be the ability of resveratrol to specifically activate sirtuin-like protein deacetylases. Sirtuins are redox-sensing enzymes that regulate a wide range of important biological processes, modulate the cellular response to environmental stresses, and allow a reduction in general metabolic activity, delayed apoptosis, and increased longevity (Halls and Yu 2008).

Currently, there is high market demand for resveratrol and the natural content of resveratrol in plants is insufficient to meet the demand of over 100 t/year (Mei et al. 2015). One option to meet the needs of the nutritional supplement industry is to utilize resveratrol derivatives (piceatannol, resveratrodehydes, and pterostilbene) in addition to plant-produced resveratrol since they may have equivalent bioactivities (Leifer and Barberio 2016; Lin et al. 2014; Martinez-Marquez et al. 2016; Wang et al. 2014). Another option is to use chemical synthesis, but this approach presents challenges because it requires a complex procedure and requires the use of toxic organic solvents and heavy metals which can result in environmental contamination (Suzuki et al. 2014). As an alternative to chemical synthesis, plant cell culture, hairy roots tissues, and metabolic engineering can be combined to biosynthesize resveratrol on a large scale and with high-yield. These methods have been shown to allow production of a wide spectrum of plant secondary metabolites with multiple bioactivities (Almagro et al. 2015; Becker et al. 2003; Krivoruchko and Nielsen 2015; Lin et al. 2014). Efforts have been made to improve the efficacy of the in vitro platform, such as the use of elicitors, introducing specific genes with different promoters, using different hosts, codon optimization, altering culture conditions, and regulating metabolic flux (Jeandet et al. 2012). Codon optimization can be performed to modify the sequence of the exogenous gene to conform its codon usage to that of the specific host (Klein-Marcuschamer et al. 2010). Compared to the synthesis of protein containing rare codons, codon-optimized sequences are more abundantly expressed in specific hosts, allowing efficient expression of exogenous gene and improved yields of metabolites (Anthony et al. 2009; Chang et al. 2007; Engels et al. 2008; Wang et al. 2011b). In this review, we highlight the latest studies on the enhancement of resveratrol bioproduction and the expression of related enzymes and discuss the contributions of different factors that contribute to maximizing production.

Biosynthesis of resveratrol in plants and microorganisms

The biosynthesis pathway of resveratrol in plants is the phenylpropanoid pathway (Fig. 2). It begins with the synthesis of phenylpropanoic acids from aromatic amino acid phenylalanine (Phe) or tyrosine (Tyr) via the shikimate pathway (Lim et al. 2011). In the following steps, there are two branches. *TAL*/*PTAL* catalyzes the desamination reaction of Tyr to form *p*-coumaric acid. Next, *4CL* combines *p*-coumaric acid and coenzyme A (CoA) to produce 4-coumaroyl-CoA. In the separate branch, cinnamic aid is formed as the product of Phe catalysis by *PAL*/*PTAL* (Becker et al. 2003; Jeandet et al. 2012; Mei et al. 2015). In some species such as *Zea mays* L., *PAL* can also use tyrosine as a substrate to produce *p*-coumaric acid (Mei et al. 2015). Cinnamic acid is then catalyzed by *C4H* and *4CL* in either order to form 4-coumaroyl-CoA. Finally, stilbene synthase (*STS*) acts to catalyze the condensation reaction of successive units of malonyl CoA with 4-coumaroyl-CoA. *STS* plays two roles in the pathway. First, *STS* combines 3 malonyl CoA to form a linear polyketide molecule skeleton. Second, *STS* directs a C2 to C7 aldol cyclization of the polyketide to form resveratrol (Chong et al. 2009; Delaunois et al. 2009; Flores-Sanchez and Verpoorte 2009).

Some endophytic fungi have the capability to produce high value bioactive molecules like their host-plants (Lou et al. 2013; Wang et al. 2014). This phenomenon may be the result of horizontal gene transfer (HGT) that occurred between

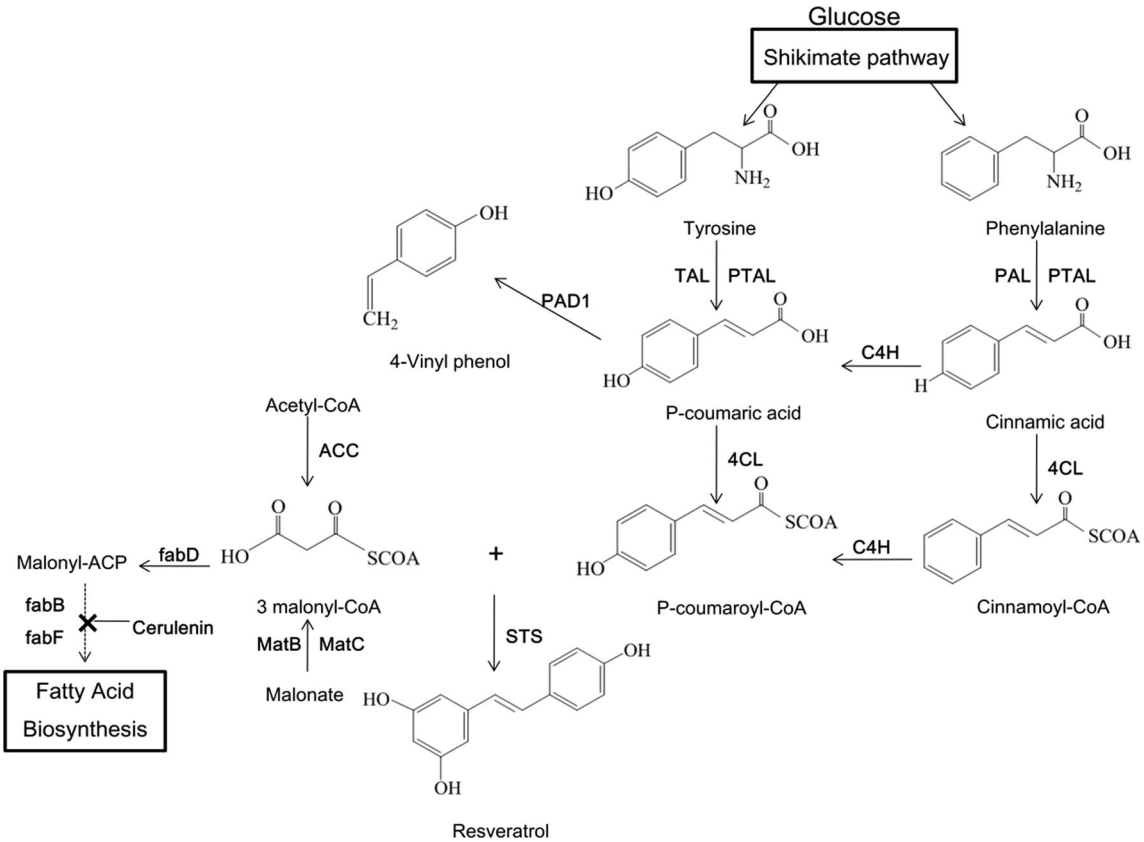

Fig. 2 Biosynthesis of resveratrol via phenylpropanoid pathway in plants and engineered microbes. *PAL* phenylalanine ammonia lyase, *PTAL* phenylalanine hydroxylase, *TAL* tyrosine ammonia lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate coenzyme A ligase, *STS* stilbene synthase, *PDA* phenyl acrylic acid decarboxylase. Increasing the pool of malonyl-CoA can be realized either by over-expressing *ACC* (acetyl-CoA carboxylase), *MatB* (malonyl-CoA synthetase) and *MatC* (malonate carrier protein) or inhibiting *fabB-fabF* of fatty acid synthesis using cerulenin

plants and microorganisms during their long period of co-evolution (Venugopalan and Srivastava 2015). Some fungal biosynthetic enzymes are homologous to their host counterparts, consistent with the HGT hypothesis. Recently, Zhang et al. (2013b)) identified the resveratrol synthesis pathway of *Alternaria* sp. MG1 (isolated from grape) by the detection of intermediates and the biosynthesis of resveratrol upon addition of Phe and Tyr, similar to the phenylpropanoid pathway in plants. However, in order to confirm the metabolic flow of resveratrol, further analysis of *Alternaria* sp. MG1 including genomic and transcriptomic studies are required.

Key enzymes contributing to resveratrol biosynthesis

PAL/TAL

Phenylalanine ammonia lyase (*PAL*, EC 4.3.1.24) is a key enzyme catalyzing the first step in the phenylpropane pathway converting Phe into *trans*-cinnamic, and thus channeling the carbon flow to synthesize important secondary metabolites such as stilbenes, flavonoids, coumarins, pinoresinol diglucoside, and lignin (Kong 2015). Its activity may increase under biotic and abiotic stresses in vivo (Świeca 2016). *PAL* has been found, isolated, and characterized in a variety of species including plants, fungi, and prokaryotes, but not in animals (Kong 2015; MacDonald et al. 2016). *PAL* also exhibits activity in the reverse reaction that catalyzes *trans*-cinnamic to form Phe, which provides pools of precursor during metabolic engineering (D'cunha et al. 1994; Qi et al. 2007; Vannelli et al. 2007). *PAL* is present as gene families in most plants, but the number of *PAL* varies considerably among different species. 40–50 and 16 genes were found in potato and grapevine, respectively, but only two were found in lemon balm and raspberry (Kumar and Ellis 2001; Weitzel and Petersen 2010).

Studies have indicated that some versions of *PAL*s possess nonoxidative deamination activity that can utilize either phenylalanine or tyrosine as substrates; these are referred to as *PTAL*, or phenylalanine/tyrosine ammonia lyase (EC 4.3.1.26) (Kong et al. 2014; Nishiyama et al. 2010). Tyrosine ammonia lyase (*TAL*, EC 4.3.1.23) can directly produce *p*-coumaroyl-CoA by deamination of tyrosine, reducing the number of reaction steps involving *C4H* in the resveratrol

synthesis pathway (Ferrer et al. 2008). *TAL* was first discovered in *Rhodobacter capsulatus* by genome analysis in 2001. It has only been identified in prokaryotes, including *Streptomyces globisporus* and *Saccharothrix espanaensis* (Watts et al. 2006). *PAL* and *TAL* are members the same family of enzymes with lyase activity which can transform amino acids into α, β-unsaturated acids by the removal of ammonia. These enzymes also have a mutase activity that can form β-amino acids (Jendresen et al. 2015). Interestingly, the substrate specificity of *PAL* and *TAL* is controlled by a single amino acid. *TAL* is unable to use Phe as its substrate, but when its His89 residue is replaced by Phe, its substrate selectivity completely switches from tyrosine to phenylalanine; the single point mutation similarly can switch the substrate specificity of *PAL* (Pinto et al. 2015).

C4H

Cinnamate 4-hydroxylase (*C4H*, EC 1.14.13.11), the key enzyme catalyzing the second step in the phenylpropanoid pathway, combines with partner reductase (*CPR*) and subsequently catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid (Luo et al. 2015). It is part of the CYP73 family of cytochrome P450 monooxygenases and is involved in the biosynthesis of a large and diverse variety of secondary metabolites (Singh et al. 2009). After it was discovered and isolated, the full-length cDNA encoding this enzyme was identified and cloned from many plants, including *Jerusalem artichoke*, mung bean, and alfalfa. *C4H* activity can be induced by a number of stresses, including chemical treatment and mechanical wound (Batard et al. 1997; Schilmiller et al. 2009).

In the phenylpropanoid metabolic pathway, *C4H* may be the rate-limiting step. Reducing the *C4H* transcriptional level leads to a 2.5-fold decrease of *4CL* and an eightfold decrease of *PAL* activities in tobacco (Li et al. 2015a). Additionally, *C4H* fails to efficiently express in *E. coli* but can be successfully expressed in *S. cerevisiae* (Achnine et al. 2004; Lee et al. 2016). Because of these limitations, *C4H* is rarely used in the construction of a heterogenous expression system of resveratrol. Instead, strategies of introducing *TAL* bypass the *C4H* catalytic step to directly produce *p*-coumaric acid.

4CL

4-Coumarate coenzyme A ligase (*4CL*, EC 6.2.1.12) was first found and identified in 1981 (Ragg et al. 1981). Subsequently, a series of *4CL*s were purified and characterized in rice, *Arabidopsis thaliana*, aspen, soybean, and other plant species (Li et al. 2014; Zhang et al. 2015a). The *4CL* gene family is much smaller compared with *STS* and *PAL* (Chen et al. 2014a). *4CL* plays an important role in catalyzing diverse aromatic substrates like *p*-coumaric acids, cinnamic acids, caffeic acids, and ferulic acids into corresponding CoA thioesters. Subsequently, these products participate in the biosynthesis of numerous secondary metabolites (Gao et al. 2015). The *4CL* genes are classified into two types: Type I and Type II. The amino acid sequences of Type I genes are more conserved and are mainly associated with lignin accumulation and Type II regulates flavonoid metabolism (Rao et al. 2014; Sun et al. 2015). Crystal structures of *4CL* showed that *4CL*s exhibit different isoform distribution patterns allowing recognition of specific substrates for the synthesis of different products (Gao et al. 2015; Li and Nair 2015b).

STS

Stilbene synthase (*STS*,EC 2.3.1.95) is a key enzyme that catalyzes the final step in the resveratrol biosynthesis pathway. *STS* belongs to the polyketide synthase (*PKS*) super family and was first purified from stressed cell suspension cultures of peanuts (*Arachis hypogaea*) (Chong et al. 2009; Delaunois et al. 2009; Vannozzi et al. 2012). It is a dimer of approximately 90 kDa molecular weight with an isoelectric point of 4.8. The genes encoding *STS* have been identified and isolated from many plant species including grapevine, peanut, pine, and sorghum. It appears to exist as a family of closely related genes in most plants except for sorghum, in which only one *STS* member has been identified (Kiselev et al. 2013b). Genomic analysis of grapevine revealed 48 *STS* genes and at least 33 full-length sequences encoding *STS* protein were obtained. These sequences were classified into three clusters based on phylogenetic tree analysis and predicted amino acid sequences (Vannozzi et al. 2012). *STS* families share 75–90 % similarity in amino acid sequence but significantly differ in the promoter regions and in the hydrogen-binding domain adjacent to the downstream regions (Duan et al. 2015; Chong et al. 2009; Lim et al. 2011; Shi et al. 2014). *STS* is predominantly located in vesicles or close to the cell wall in plant tissues and is associated with resveratrol excretion (Fornara et al. 2008).

Three residues in the active site (Cys164, His303, and Asn336) are conserved in all known *STS*s. The active site is composed of a substrate-binding pocket, a CoA-binding tunnel, and a cyclization pocket (Flores-Sanchez and Verpoorte 2009). This specific protein conformation can catalyze diversified cyclization reactions. In flavonoid biosynthesis, the C6 and C1 of backbone are connected directly through a Claisen condensation reaction by chalcone synthase (*CHS*). However, for stilbenes, cyclization occurs as an aldol type of condensation between C2 and C7 with an additional decarboxylative loss of CO_2 in the position of C1. The mechanistic differences between these two reactions can be explicated by their crystal structures. The presence of a thiolase fold and a cryptic thioesterase activity switches the cyclization reaction from Claisen condensation (*CHS*) to Aldol condensation (*STS*) (Ferrer et al. 2008). Overall, knowledge of resveratrol

metabolism and the function of key enzymes in the pathway will lay the foundation for enhancing resveratrol biosynthesis efficiency via metabolic engineering.

Metabolic engineering—a promising method for the biosynthesis of resveratrol in vitro

In the past five decades, extensive studies of the synthesis and metabolism of resveratrol have led to multiple strategies for the production of resveratrol, including plant and microorganism resources, chemosynthesis, plant cell culture, metabolic engineering, and other methods (Almagro et al. 2015; Cai et al. 2012b; Kang et al. 2015; Krivoruchko and Nielsen 2015; Zhang et al. 2013a; Zhang et al. 2013b). Plants are the major natural resources for producing resveratrol, but its biosynthesis is limited to only a few species such as *Polygonum cuspidatum*, *Vitis vinifera*, peanut, and berry fruits. These plants contain extremely low content of resveratrol and typically produce resveratrol only under some biotic and abiotic stresses (Chung et al. 2003; Zamboni et al. 2006). In grapes, resveratrol is mainly accumulated in the skin of grape berries at a level of approximately 1.5–7.8 mg/g fresh weight (FW) (Mei et al. 2015), while the detected concentration is 2 and 50.61 μg/g dry weight (DW) in peanut and mulberry fruit, respectively (Hasan et al. 2012; Nopo-Olazabal et al. 2013; Sales and Resurreccion 2014; Shrikanta et al. 2015). Additionally, trace amounts of resveratrol have been found in some food products, such as chocolate and cocoa liquor (Counet et al. 2006; Jeffrey Hurst et al. 2008; Jerkovic et al. 2010; Pimentel et al. 2010). Although some new technologies have been developed to improve the extraction of resveratrol from plants, it remains inefficient, costly, and unable to meet the market demand (He et al. 2015; Lu et al. 2015; Soural et al. 2015).

The chemical synthesis of resveratrol may be able to achieve the goal of relatively high yields. However, it presents disadvantages in the production of byproducts during the complex process and the requirement for toxic organic solvents which can result in environmental contamination (Du et al. 2011; Fan et al. 2010).

The use of plant cell cultures has been widely applied for the production of resveratrol due to the low-cost of medium (Jeandet et al. 2014). In fact, this method has inherent advantages that allow the constitutive production of resveratrol and the yield can be increased in response to stresses (Donnez et al. 2009). A grapevine *V. vinifera* L. cv. Monastrell Albino cell suspension produced 5207 mg/L resveratrol when randomly methylated-beta-cyclodextrin was added as an elicitor (Martinez and Garcia 2007). The use of *V. vinifera* cell suspension with elicitors-cyclodextrin or cyclodextrin derivatives could allow synthesis of resveratrol in a range of 700–5000 mg/L (Kiselev 2011). However, the main limiting factor of plant cell culture is genomic instability which can give rise to silencing of some gene clusters (Venugopalan and Srivastava 2015). Moreover, products released in the culture media may be susceptible to enzyme degradation (Cai et al. 2012a).

Considering the above technologies, metabolic engineering may be the most promising and eco-friendly approach. *E. coli* and *S. cerevisiae* are popular choices for in vitro platforms for resveratrol synthesis and have advantages including fast growth rate, simple and inexpensive culture medium, and ease of genetic manipulation (Wang et al. 2016). The *S. cerevisiae* expression system provides a number of distinct advantages over that in *E. coli*. First of all, *S. cerevisiae* is a eukaryotic organism and thus expresses some complex enzymes (*C4H*) more efficiently and stably than *E. coli*. Additionally, *S. cerevisiae* exhibits higher resistance to low pH and high osmotic stress (Wang et al. 2011a). Finally, it belongs to the class of generally regarded as safe (GRAS) microorganisms, allowing its use in food fermentation to improve nutritional value (Kong 2015; Wang et al. 2016). The development of metabolic engineering tools allowed the successful reconstitution of resveratrol heterologous pathway in both *E. coli* and *S. cerevisiae*. Engineered strains can produce resveratrol with yields of 531.41 and 2300 mg/L in *S. cerevisiae* and *E. coli*, respectively (Li et al. 2015a; Lim et al. 2011). Notably, the engineered plasmid can also be transformed into other hosts in addition to the two commonly used above. Kang et al. (2015) first transformed an expression vector with *4CL* and the resveratrol synthase gene (*RS*) into bTf (blastospore of *Tremella fuciformis*) by LiAc/PEG-mediated transformation. The transformant can produce 0.92 μg/g DW resveratrol upon the addition of *p*-coumaric acid as a substrate. Tantong et al. (2016) successfully selected a strain of cyanobacterium *Synechocystis* PCC 6803 to engineer a heterologous pathway of resveratrol with *TAL*, *4CL*, and *STS* genes. In addition to introducing exogenous genes, gene replacement into a specific location was reported to allow successful synthesis of resveratrol in *E. coli*. Liu et al. (2016) utilized the site-specific replacement strategy to integrate *STS*, *TAL*, and *4CL* into the loci of genes tyrR and trpED in the chromosome of *E. coli* BW25113 to produce 4.612 mg/L resveratrol.

Recently, there are reports of resveratrol synthesis using novel approaches. Zhang et al. (2013a) used resting cells of a non-genetically modified strain, *Alternaria* sp. MG1, to produce 1.376 μg/L resveratrol from phenylalanine. Another new approach is enzymatic transformation

that allows synthesis of resveratrol from polydatin by glucosidase (Chen et al. 2014b; Zhang et al. 2014).

Strategies for enhancing resveratrol production and pathway enzymes

Introducing the whole metabolic pathway or specific enzyme-encoding genes

In order to improve resveratrol production and upregulate the expression of key enzymes, it is crucial to construct a resveratrol biosynthesis pathway via introduction of the entire pathway or specific genes with the initial addition of corresponding substrates (phenylalanine and tyrosine). Shin et al. (2012) obtained 3.3 mg/L resveratrol by using recombinant yeast with *PAL*, *C4H*, *4CL*, and *STS* genes grown in YP medium (1 % yeast extract and 2 % peptone) containing 2 % galactose. The yield was further increased to 5.8 mg/L when tyrosine was added. Similarly, Wu et al. (2013) established an *E. coli* system by adding the whole pathway genes to obtain resveratrol at a level of 35.02 mg/L. Interestingly, the capacity of synthesizing resveratrol from different exogenous expressing systems varies considerably due to several factors, including gene resources, the choice of promoters, host strains, vector designs, addition of substrates, and the optimization of culture growth conditions (Lim et al. 2011; Shi et al. 2014; Sydor et al. 2010; Wu et al. 2013). For example, a recombinant yeast strain transformed with *STS* and *4CL* genes produced 6 mg/L resveratrol after 144 h of incubation in SD medium (6.7 g/L yeast nitrogen base with amino acids, 20 g/L glucose) supplemented with 5 mM *p*-coumarate. Then after switching to rich YEPD medium (10 g/L yeast extract, 20 g/L bacterial peptone, 20 g/L glucose) with geneticin and hygromycin supplemented with 10 mM *p*-coumarate, the strain was able to achieve 262 mg/L resveratrol (Sydor et al. 2010). However, it may be easier to enhance the resveratrol content in plant cells (including hairy root culture) as this approach would not require genetic engineering.

Use of elicitors

Elicitors are signaling molecules that activate plant defense mechanisms and function through a massive reprogramming of gene expression that allows both the activation of resveratrol biosynthesis and the expression of related proteins (Almagro et al. 2015). Elicitors can be classified as biotic and abiotic, and the former includes polysaccharides, proteins, and oral secretions from insects and the latter includes UV-light, metal ions, and chemical compounds (Cai et al. 2013; Laura et al. 2007; Yang et al. 2015). The improvement of resveratrol synthesis in plant cell cultures triggered by elicitors is well-documented (Almagro et al. 2015; Belchi-Navarro et al. 2012; Keskin and Kunter 2008). Here, we discuss the latest findings about elicitors and their action mechanisms. The improvements in resveratrol content and the expression of key enzymes by different elicitors are presented in Table 1.

Cyclodextrins, jasmonic acid (JA), and its methyl ester, methyl jasmonate (MeJa) are effective elicitors involved in the signal transduction pathway and induce the biosynthesis of resveratrol as a component of plant defense reactions (Belchi-Navarro et al. 2012; Dubrovina and Kiselev 2011; Vuong et al. 2014). With a similar mechanism to that of cyclodextrins and JA, coronatine (including its analog indanoyl-isoleucine) and salicylic acid (SA) can also induce synthesis of secondary metabolites and upregulate the expression of pathway enzymes (Almagro et al. 2015; Kiselev et al. 2013a). Kiselev et al. (2015) found that resveratrol production increased by almost fivefold with the use of SA, resulting in increased expression of all *STS* genes except for *STS7*. Other studies revealed that elevated levels of cytoplasmic calcium could stimulate resveratrol production. A calcium ionophore, A23187 was found to increase resveratrol content and the expression of *STS* by influencing calcium influx (Kiselev et al. 2012).

Actually, biosynthetic gene clusters are often transcriptionally controlled by epigenetic regulation including methylation and acetylation (Venugopalan and Srivastava 2015). They are associated with gene silence and expression, thereby involving the synthesis of secondary metabolites. Recently, a study found that when 5-azacytidine (azaC) and chitosan were used to treat plant cells, the amount of resveratrol increased two to three times and the expression of biosynthetic genes upregulated greatly. AzaC is a demethylation agent that can significantly decrease the methylation level of DNA and chitosan can alter the acetylation level (Kiselev et al. 2013b; Xu et al. 2015a). Interestingly, SA also decreased the methylation of *STS2* and *STS10* genes when it was used to enhance resveratrol production (Kiselev et al. 2015). Additionally, UV radiance, well-known to induce resveratrol synthesis in plants, is thought to be connected with the demethylation of *STS* genes as well (Tyunin and Kiselev 2015).

Metal ions act as abiotic elicitors and induce biosynthesis of resveratrol in plant cell cultures. Co^{2+} and Ag^{+} at different concentrations (5, 25, and 50 μM) were employed to stimulate resveratrol production in *V. vinifera*, resulting in 1.3–1.5-fold increase in the yields of resveratrol for all treatments (Cai et al. 2013). Ag^{+} also can promote other secondary metabolites, such as tanshinones (Li et al. 2016; Zhao et al. 2010). Additionally, Cd^{2+} has a significant effect in improving resveratrol production and is widely used to treat grapevine cell cultures. Cd^{2+} can increase the yields of resveratrol 3.5–4.2-fold (Cetin et al. 2014; Çetin and Baydar 2016). However,

Table 1 Examples of elicitor addition for enhancement in resveratrol yield and enzymes expression

Plant Cells	Elicitors	Resveratrol quantity[a]	Resveratrol quantity[b]	Enzymes expression	Promoters[c]	References
V. vinifera L. cv. Monastrell calli	Cyclodextrins 50 mM	ND	201 mg/L	*PAL*, *STS*, *4CL*,*C4H* ↑		Almagro et al. 2015
	Cyclodextrins 50 mM + Coronatine 1 μM	ND	684 mg/L	*PAL*, *STS*, *4CL*,*C4H* ↑		
	Coronatine 1 μM	ND	ND	*PAL*, *STS*, *4CL*,*C4H* ↑		
V. vinifera cv. Pinot Noir calli	DIMEB 50 mM	ND	0.51 mg/L			Zamboni et al. 2006
V. vinifera cv. Merzling calli	DIMEB 50 mM	0.49 mg/L	4.31 mg/L			
Vitis amurensis calli	DIMEB 50 mM	0.31 mg/L	225.22 mg/L			
Vitis riparia × *Vitis berlandieri* calli	DIMEB 50 mM	0.37 mg/L	911.25 mg/L			
V. amurensis Rupr. calli	SA 300 μM	0.6 mg/L	3.4 mg/L	*STS1–6,8–10* ↑		Kiselev et al. 2015
V. vinifera L. cv. Gamay Fréaux calli	In-Ile 8 mg/L	1.3 μmol/L	3.16 μmol/L			Cai et al. 2012b
V. vinifera L. cv. Gamay Fréaux calli	Lin-Gln 8 mg/L	1.3 μmol/L	3.59 μmol/L			
V. vinifera L. cv. Gamay Fréaux calli	Insect saliva (*Manduca sexta* larvae) 1.4 mg/L	1.3 μmol/L	5.25 μmol/L			
V. amurensis Rupr. calli	Sodium nitroprusside 0.1 mM	0.036 % DW	0.150 % DW			Kiselev et al. 2007
V. vinifera cv. Regent calli	Autoclaved culture filtrate of *Aureobasidium pullulans*	5 μg/g DW	200 μg/g DW	*PAL*, *STS* ↑		Ruhmann et al. 2013
V. vinifera L. cv. Gamay Fréaux cell line	β-glucan 1 mg/L	3.8 mg/L	8.0 mg/L			Vuong et al. 2014
	JA 20 μM	3.8 mg/L	17.0 mg/L			
	β-glucan 1 mg/L + JA 20 μM	3.8 mg/L	13.0 mg/L			
	SA 100 μM	3.8 mg/L	12.5 mg/L			
	SA 100 μM + JA 10 μM	3.8 mg/L	8.0 mg/L			
	Chitosan 0.5 mg/L	3.5 mg/L	9.3 mg/L			
V. vinifera L. Cabernet Sauvignon calli	UV light 254 nm	0.85 μg/g FW	60.56 μg/g FW			Keskin and Kunter 2008
V. vinifera L. cv. Monastrell calli	MeJa 100 μM	ND	16.4 μmol/g DW			Belchi-Navarro et al. 2012
	MeJa 100 μM + Cyclodextrins 50 mM	338.6 μmol/g DW	1447.8 μmol/g DW			
V. amurensis Rupr. calli	5-Azacytidine 200 μM	2 mg/L	5 mg/L	*STS10* ↑		Kiselev et al. 2013b
V. amurensis Rupr. calli (*rolB*)	5-Azacytidine 200 μM	16 mg/L	31 mg/L	*STS10* ↑	CaMV 35S promoter	
V. amurensis Rupr. calli	Calcium ionophore 1 μM	<0.02 % DW	0.04 % DW	*STS1–6*, *10* ↑		Kiselev et al. 2012
V. amurensis Rupr. calli	SA 100 μM	0.581 mg/L	3.478 mg/L	*STS2*, *4–7*, *10*↑		Kiselev et al. 2013a
	Phenylalanine 100 μM	0.581 mg/L	1.204 mg/L	*STS8–10* ↑		
	Coumaric acid 100 μM	0.581 mg/L	1.419 mg/L	*STS1–8*,*10* ↑		

Table 1 (continued)

Plant Cells	Elicitors	Resveratrol quantity[a]	Resveratrol quantity[b]	Enzymes expression	Promoters[c]	References
V. amurensis Rupr. calli	UV-C 254 nm	0.0055 % DW	0.0412 % DW	*STS1–10*, *PAL*1,4 ↑		Tyunin and Kiselev 2015
V. vinifera L. cv. Cabernet Sauvignon calli	Chitosan 50 mg/L + UV-C 254 nm	0.5 mg/L	3.05 mg/L	*PAL*,*C4H*,*4CL*, *STS* ↑		Xu et al. 2015a
V. amurensis Rupr. calli (*rolB*)	SA 150 μM	0.5 % DW	2 % DW		CaMV 35S promoter	Dubrovina and Kiselev 2011
	MeJa 50 μM	0.5 % DW	0.83 % DW			
V. amurensis Rupr. calli	Coumaric acid 0.1 mM	0.35 mg/L	3.6 mg/L	*PAL1*,*2 STS2*,*9* ↑		Shumakova et al. 2011
V. amurensis Rupr. calli (*rolB*)	Coumaric acid 0.5 mM	26.73 mg/L	40.1 mg/L	*PAL2*,*4 STS2*,*3* ↑	CaMV 35S promoter	
V. vinifera L. cv. Gamay Fréaux calli	Co^{2+} 5 μM	152.4 μmol/g DW	216.8 μmol/g DW			Cai et al. 2013
	Ag^{2+} 50 μM	152.4 μmol/g DW	233.0 μmol/g DW			
V. vinifera L. cv. Barbera calli	Na-orthovanadate 1 mM	70 nmol/g DW	100 nmol/g DW			Tassoni et al. 2005
V. vinifera L. cv. KALECİK KARASI calli	$CdSO_4$ 1.5 mM	0.851 μg/g FW	3.650 μg/g FW			Çetin and Baydar 2016
	MeJa 10 μM	0.473 μg/g FW	2.872 μg/g FW			
	Sucrose 0.20 M	2.491 μg/g FW	7.550 μg/g FW			
V. vinifera L. cv. ÖKÜZGÖZÜ calli	MeJa 10 μM	4.095 μg/g FW	11.681 μg/g FW			
V. vinifera L. cv. ÖKÜZGÖZÜ calli	$CdCl_2$ 1.0 mM	1.392 μg/g FW	4.908 μg/g FW			Cetin et al. 2014
V. vinifera L. cv. Cabernet Sauvignon calli	Oligochitosan 100 mg/L	<200 μg/g DW	410 μg/g DW	*PAL*,*C4H*,*4CL*, *STS* ↑		Xu et al. 2015b
	Sodium alginate 100 mg/L	<200 μg/g DW	300 μg/g DW	*PAL*,*C4H*,*4CL*, *STS* ↑		

ND not detection, ↑ upregulate, *DW* dry weight, *FW* fresh weight, *DIMEB* dimethyl-β-cyclodextrin, *SA* salicylic acid, *In-Ile* indanoyl-isoleucine, *Lin-Gln* n-linolenoyl-l-glutamine, *JA* jasmonic acid, *MeJa* methyl jasmonate, *rolB* transformation with *rolB* gene, *CaMV 35S promoter* cauliflower mosaic virus 35S promoter

[a] Indicates the quantity of resveratrol before treatment of elicitor

[b] Indicates the quantity of resveratrol after treatment of elicitor

[c] Indicates that *rolB* gene is expressed under the control of the cauliflower mosaic virus 35S promoter

these heavy metals had a negative effect on cell growth and inhibited cell viability remarkably except for Co^{2+} (Cai et al. 2013; Cetin et al. 2014). It has been demonstrated that cadmium inhibits and disturbs various physiological and biochemical processes such as respiration, photosynthesis, cell elongation, nitrogen metabolism and mineral nutrition when it is excess in plants, resulting in cell death and inhibition of growth (Çetin and Baydar 2016). Although little is known about the defense mechanism of grapevine cell cultures responding to metal iron stresses, it has been found that Cd^{2+} significantly increased *PAL* activity more than 25-fold on day 3 when compared to control cells (Cetin et al. 2014). Similarly, Ag^{+} treatment remarkably upregulated the expression of key enzymes of the phenylpropanoid pathway including *PAL*, *C4H*, and *4CL* almost 5–30-fold (Xing et al. 2015). Therefore, we speculate that metal irons may be involved in the phenylpropanoid pathway to cause enhancement of resveratrol production.

Another approach to increase resveratrol production is feeding the plant cells precursors of resveratrol. These precursors are key components of the phenylalanine pathway, important for synthesizing resveratrol (Shin et al. 2011). However, the influence of precursors on resveratrol biosynthesis is concentration-dependent (Kiselev et al. 2013a; Shumakova et al. 2011).

Engineer of non-pathway exogenous genes

The aim of engineering a tailored vector with non-pathway genes is to elevate the intracellular concentration of precursors, regulating synthetic pathway, increasing the activity of key enzymes, and promoting resveratrol release and accumulation. Successful examples of enhancing resveratrol production using these kinds of strategies are summarized in Table 2.

The agrobacterium *rolB* gene is a powerful tool for induction of resveratrol synthesis in transgenic strains and cultured plant cells. The over-expression of *rolB* gene resulted in more than a 100-fold increase in resveratrol production in *V. amurensis* Rupr. callus culture compared to the control (Kiselev et al. 2007). The positive influence of *rolB* gene on resveratrol accumulation was demonstrated to be Ca^{2+}dependent. Dubrovina et al. (2009) added calcium channel blockers, $LaCl_3$, verapamil, and niflumic acid to treat *rolB* transgenic cultures and found that resveratrol production decreased significantly. Taken together, these reports suggest that the flux of Ca^{2+} and signal transduction mediated by Ca^{2+} *are* necessary to increase of resveratrol production. When *VaCPK20* gene, encoding a calcium-dependent protein kinase (CDPK) was transformed into five cell lines of *V. amurensis* under the control of CaMV 35S promoter, it resulted in 9–68 times improvement in resveratrol production and the upregulation of the *STS7* gene (Aleynova-Shumakova et al. 2014). However, not all members of the *CDPK* multigene family have promoting effect on resveratrol accumulation. Aleynova et al. (2015) investigated the influence of overexpression of four *CDPK* genes (*VaCPK9*, *VaCPK13*, *VaCPK21*, and *VaCPK29*) on resveratrol biosynthesis and found that only *VaCPK29* overexpression increased resveratrol content 1.6–2.4-fold but there was no obvious effect on resveratrol biosynthesis of overexpression of the other genes. Because CDPKs are major sensors of Ca^{2+}, the high level of intracellular calcium plays an important role in resveratrol synthesis.

In order to increase precursor levels of phenylpropanoid pathway and accelerate resveratrol synthesis, the introduction of non-pathway genes is very important (Fig. 2). Acetyl-CoA carboxylase (*ACC*) is a key enzyme in the acetate assimilation pathway which catalyzes acetyl-CoA into malonyl-CoA and subsequently elevates the cytosolic malonyl-CoA pool. However, the over-expression of *ACC* in *S. cerevisiae* only resulted in a slight improvement in resveratrol production, one to twofold (Li et al. 2015a; Shin et al. 2012). This may be because malonyl-CoA is also the precursor of other secondary metabolites, such as flavonoids. Enhancement of resveratrol content may require overexpression of both malonyl-CoA synthetase (*matB*) and malonate carrier protein (*matC*) with addition of malonate to increase the intracellular levels of malonyl-CoA and inhibiting fatty acid synthesis using cerulenin (Lim et al. 2011; Wang et al. 2011a). Overexpression of upstream genes DAHP-synthase (*ARO4*) and chorismate mutase (*ARO7*) in the prephenic acid pathway increased the amounts of phenylalanine precursor. Applying this strategy allowed resveratrol production to increase to 4.85 mg/L (Li et al. 2015a). Surprisingly, knockout of the phenyl acrylic acid decarboxylase (*PAD1*) gene did not improve the conversion of *p*-coumaric acid to resveratrol; further studies are required to understand this effect (Shin et al. 2011).

V-myb myeloblastosis viral oncogene homolog (MYB) transcriptional factors act as key regulators of the synthesis of phenylpropanoid-derived compounds (Liu et al. 2015). MYB proteins are classified into four groups: 1R, 2R, 3R, and 4R. 2R proteins are mainly involved in secondary metabolism and the cellular response to biotic and abiotic stresses. Höll et al. (2013) identified two 2R-MYB transcriptional factors (TFs) from grapevine, *MYB14* and *MYB15*, which were demonstrated to specifically activate the *STS* promoters. Additionally, the ectopic expression of *MYB15* in grapevine hairy roots resulted in increased *STS* expression and the accumulation of resveratrol. Moreover, the average expression of the *PAL* gene in *MYB15* trangenic lines was 13-fold higher than that of the control. Recently, the role of MYB TFs to regulate the resveratrol biosynthetic pathway was further confirmed by Fang et al. (2014). Transient overexpression of *MYB14* in young "Jingzaojing" (*V. vinifera*) leaf could promote the expression of *STS* 15-fold. They also found that *MYB14* proteins could recognize the promoter of *STS* and

Table 2 Examples of introducing non-pathway genes for enhancement in resveratrol yield and enzymes expression

Microorganisms/plant cells	Non-pathway genes/species	Resveratrol quantity[a]	Resveratrol quantity[b]	Enzymes expression	Promoters[c]	Reference
V. amurensis Rupr. callus	*VaCPK20* gene/inflorescence (*V. amurensis*)	0.008 % DW	0.043–0.418 % DW	*STS7* ↑	CaMV 35S promoter	Aleynova-Shumakova et al. 2014
V. amurensis Rupr. callus	*VaCPK29* gene/ inflorescence (*V. amurensis*)	0.008 % DW	0.013–0.019 % DW		CaMV 35S promoter	Aleynova et al. 2015
S. cerevisiae (*4CL::STS*)	*araE*/*E. coli*	1.27 mg/L	3.1 mg/L			Wang et al. 2011b
S. cerevisiae (*TAL*, *4CL*, *RS*)	*ARO4* + *ARO7*/*S. cerevisiae*	2.73 mg/L	4.85 mg/L			Li et al. 2015a
	ACC1/*S. cerevisiae*	2.73 mg/L	3.57 mg/L			
	ARO4 + *ARO7* + *ACC1*/*S. cerevisiae*	2.73 mg/L	6.39 mg/L			
V. vinifera cv. Chardonnay hairy roots	*MYB15*/grape berry cDNA (Shiraz)	0.25 nmol/g FW	1.8 nmol/g FW	*STS*, *PAL* ↑	CaMV 35S promoter	Höll et al. 2013
S. cerevisiae (*4CL*, *STS*)	*PAD1* deletion	3.1 mg/L	2.8 mg/L			Shin et al. 2011
S. cerevisiae (*PAL*, *C4H*, *4CL*, *STS*)	*ACC1*/*S. cerevisiae*	3.3 mg/L	4.3 mg/L		GAL1 promoter	Shin et al. 2012
S. cerevisiae	*bgIN*/*Candida molischiana*	0.743 μM	1.978 μM		Actin gene promoter	Gonzalez-Candelas et al. 2000
	abfB/*Aspergillus niger* + *bgIN*/*Candida molischiana*	0.743 μM	2.066 μM		Actin gene promoter	
V. amurensis Rupr. callus	*rolB*/*Agrobacterium rhizogenes*	0.026 % DW	3.15 % DW		CaMV 35S promoter	Kiselev et al. 2007

DW dry weight, *FW* fresh weight, ↑ upregulate, *VaCPK20/VaCPK29* encoding a calcium-dependent protein kinase, *araE* encoding the low-affinity high-capacity transporter, *ARO4* encoding DAHP-synthase, *ARO7* encoding chorismate mutase, *MYB15* encoding V-myb myeloblastosis viral oncogene homolog protein, *bgIN* encoding a β-glucosidase, *abfB* encoding an α-L-arabinofuranosidase, *CaMV 35S promoter* cauliflower mosaic virus 35S promoter, *GAL1 promoter* yeast GAL1 promoter

[a] Indicates the quantity of resveratrol before non-pathway gene transformation

[b] Indicates the quantity of resveratrol after non-pathway gene transformation

[c] Indicates that the non-pathway gene transformed is expressed under the specific promoter

bind to the Box-L5 motif (gagttggtgaga), which was identified within the promoter of *STS*. Therefore, *MYB14* is directly involved in the regulation of the expression of *STS*.

Spatial organization of enzymes

Generally, most exogenous genes encoding enzymes show relatively low or no activity on their natural substrates in genetically modified strains (Lim et al. 2011). An emerging strategy to combat this problem is to co-localize pathway enzymes into multi-protein complexes through artificially scaffolded proteins or unnatural protein fusion. The advantages of this strategy include channeling intermediates and bringing active sites together, thereby increasing enzyme efficiency and improving the yield of products. For fusion protein technology, two or more genes can be connected with an amino acids linker. The activity of the fusion enzyme directly depends on the type and length of linker (Wang et al. 2011c). Zhang et al. (2015b) used a 15-amino acid flexible linker to connect the *4CL* gene (*Arabidopsis thaliana*) and *RS* gene (*Arachis hypogaea*) and produced 80.524 mg/L resveratrol when 1 mM *p*-coumaric acid was supplied. Similarly, a 15-fold increase of resveratrol was produced from an expression vector encoding a fusion protein of *4CL* (*Arabidopsis thaliana*) and *STS* (*Vitis vinifera*) connected with a three-amino acid liker (Zhang et al. 2006). An additional advantage of a fusion enzyme strategy is that it can reduce the number of vectors required in a heterologous expression system. However, protein scaffold may be more effective in increasing resveratrol synthesis and enzyme activity than the use of protein fusions. Wang and Yu (2012) cloned nine scaffold configurations consisting of the GTPase binding domain (GBD), the Src homology 3 domain (SH3), and the PSD95/DlgA/Zo-1 domain (PDZ) of different numbers into nine yeast expression vectors. These protein interaction domains were linked together by flexible nine-residue glycine-serine linkers (Dueber et al. 2009). The domains SH3 and PDZ were used to recruit *4CL* and *STS*, respectively. By optimizing the number of repeats of these domains, they obtained a more than fivefold improvement of resveratrol (6.7 mg/L) relative to the control via using the optimal scaffold ($GBD_1SH3_2PDZ_4$). Additionally, they found a 2.7-fold increase over the previous results using a fusion protein approach. These results indicate that optimization of the organization of enzymes in metabolic engineering is a promising strategy allowing improved production.

Fungal interspecific interactions

The interspecific interactions between different fungi could enhance biosynthesis of phenylpropanoid metabolites. This effect may depend on the signaling pathway mediated by nitric oxide (NO) that is generated in the co-culture system. Zhao et al. (2015) co-cultured two white-rot fungi, *Phellinus morii* and *Inonotus obliquus*, and the system induced NO synthesis that was followed by an increase in the transcriptional level of *PAL* and *4CL*. When the NO synthase (*NOS*) selective inhibitor aminoguanidine (AG) was added into the culture, NO production was significantly inhibited, eliminating transcription of *PAL* and *4CL*. Additionally, they detected upregulated biosynthesis of styrylpyrone polyphenols. Notably, the function of NO is also confirmed by Vandelle et al. (2006). They found that the production of NO that was induced in endopolygalacturonase 1 (BcPG1)-treated grapevine cells activated the transcription of both *PAL* and *STS* genes. When they added 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) into culture system to scavenge NO, reductions of the transcript accumulation of two genes were observed after a 3-h treatment. Taken together, NO involves in the expression of resveratrol synthesis genes. Therefore, we speculate that the co-culture of resveratrol producing endophytic fungus like *Alternaria* and other fungi species may generate a similar effect.

Conclusions

Many approaches have been developed for improving resveratrol production by over-expressing key enzymes using an in vitro platform. In plant cell cultures (including hairy root cultures), *rolB* gene transformation and the addition of elicitors can enhance resveratrol production. However, metabolic engineering in *E. coli* and *S. cerevisiae* is relatively complicated and requires the construction of the whole resveratrol biosynthesis pathway or introduction of specific gene with the initial addition of precursors. To increase the efficiency of recombinant microorganism, host strains, vectors, promoters and culturing conditions should be optimized. The introduction of non-pathway genes, increasing the proximity of enzymes, and promoting fungal interspecific interactions are additional strategies to enhance resveratrol production by improving enzyme activity and regulating metabolic flux.

Acknowledgment This review is supported by the National Key Technology R&D Program (No. 2015BAD16B02), the National Natural Science Fund (Grant No. 31471718), the Agriculture Department of China (Grant No. CARS-30), and the Northwestern Polytechnical University (No. 3102014JCQ15011 and No. 3102014GEKY1010).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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