Plant Foods Hum Nutr (2012) 67:191–199
DOI 10.1007/s11130-012-0299-8

ORIGINAL PAPER

Resveratrol Biosynthesis: Plant Metabolic Engineering for Nutritional Improvement of Food

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Published online: 10 July 2012

Abstract The plant polyphenol *trans*-resveratrol (3, 5, 4′-trihydroxystilbene) mainly found in grape, peanut and other few plants, displays a wide range of biological effects. Numerous *in vitro* studies have described various biological effects of resveratrol. In order to provide more information regarding absorption, metabolism, and bioavailability of resveratrol, various research approaches have been performed, including *in vitro*, *ex vivo*, and *in vivo* models. In recent years, the induction of resveratrol synthesis in plants which normally do not accumulate such polyphenol, has been successfully achieved by molecular engineering. In this context, the ectopic production of resveratrol has been reported to have positive effects both on plant resistance to biotic stress and the enhancement of the nutritional value of several widely consumed fruits and vegetables. The metabolic engineering of plants offers the opportunity to change the content of specific phytonutrients in plant - derived foods. This review focuses on the latest findings regarding on resveratrol bioproduction and its effects on the prevention of the major pathological conditions in man.

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Keywords Resveratrol · Metabolic engineering · Functional foods · Biological activity

Introduction

Recently, the interest in antioxidants without vitamin activity, such as stilbenes, has been increasing. As far as concerns stilbenes, resveratrol has been suggested to play an important role in the prevention of a number of pathological conditions in man, such as cardiovascular diseases, neurodegenerative disorders [1] and carcinogenesis [2]. Resveratrol also inhibits platelet aggregation and exhibits antioestrogenic activity [3–5]. Epidemiological studies confirmed the positive effects of a diet rich in this type of antioxidant in overcoming some degenerative disorders caused by early ageing of the cells [6–8]. However, resveratrol together with many of the best-characterized health-promoting phytochemicals are absent or present only at low levels in edible plants or plant-derived products. Therefore, an improvement in these levels by means of manipulation of secondary plant metabolism is currently being attempted to ensure adequate dietary intake of these nutrients and increase their consumption [9–12].

Chemical Properties and Biosynthesis of Resveratrol in Plants

Resveratrol and its 3-*O*-*β*-D-glucoside derivative share a chemical structure similar to that of other polyphenol-type stilbens (Fig. 1). In plant tissues, its production is either constitutive or inducible and is controlled by the key enzyme, stilbene synthase (STS), which belongs to a multigene family of the type III group of the polyketide synthase

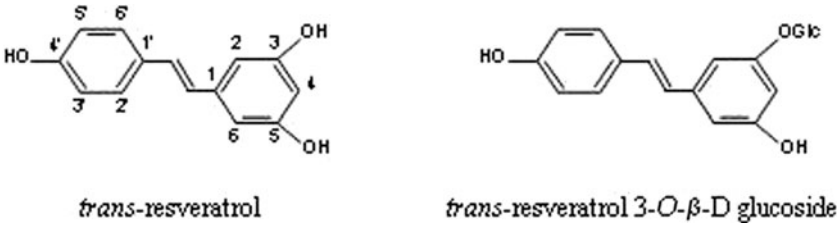

Fig. 1 Chemical structures of stilbenes

superfamily [13, 14]. STS catalyses the condensation of three molecules of coumaroyl-CoA to form resveratrol. The synthesis of resveratrol takes place in a single enzymatic step with CoA-esters of cinnamic acid derivatives and three malonyl-CoA units as starting blocks. STS and chalcone synthase (CHS) are key enzymes of the flavonoid biosynthesis pathway (Fig. 2). These enzymes, competing for the same substrates, share a high degree of homology and both contain an essential cysteine residue, namely Cys 164, considered as their active site.

The synthesis of resveratrol is strongly enhanced by fungal attacks, UV irradiation and other environmental stress conditions [13–15]. Resveratrol levels peak approximately 24 h after stress exposure, and decrease after 42–72 h as a result of activation of stilbene oxidase [16, 17].

Resveratrol exists in two stereoisoforms with configuration *cis*- or *trans*-, the latter being the most widely studied, although *cis*-resveratrol may also possess health promoting properties. In nature, the most abundant form of resveratrol would appear to be the 5,3,4′-dihydroxystilbene-3-*O*-*β*-D-glucopyranoside. The number as well as the position of moieties play an important role in the biological activity of the compound [6–18].

Edible Plants Sources of Resveratrol

Stilbenes are small naturally occurring phenolic compounds found in a wide range of plant – derived food; among which, berries, such as deerberry, cowberry, blueberry, and lingonberry, are important sources [19, 20]. Resveratrol is biosynthetically correlated to stilbenes, but its biosynthesis is restricted to only a few plant species commonly used for human consumption among which are pine, peanuts, grapes, bilberry, and mulberry [13–17, 21, 22].

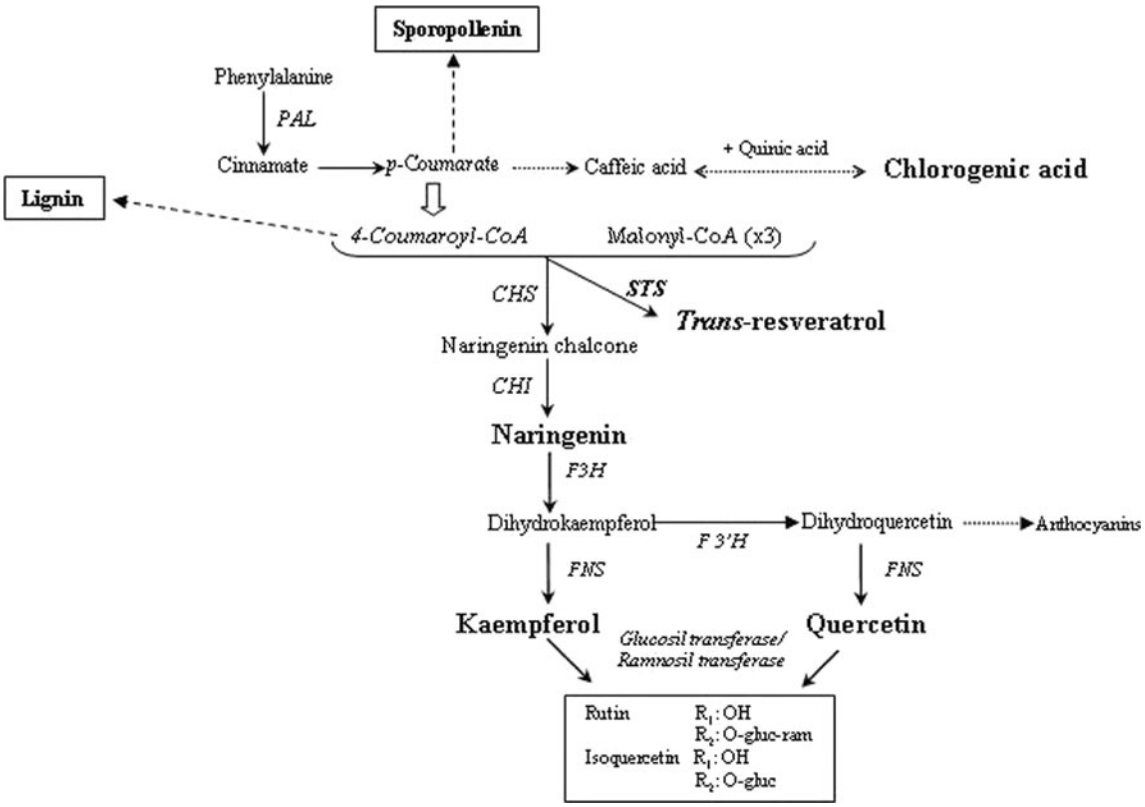

Fig. 2 Anthocyanins biosynthetic pathway. The expression of a stilbene synthase gene in transgenic plants competes with substrates used in the first step of the complex route of anthocyanins

The most abundant levels of naturally occurring resveratrol are found in the roots of Japanese Knotweed *(Poligonum cuspidatum)*, which has been used in traditional Asian herb medicine for hundreds of years in the treatment of inflammation [15]. Grape is probably the most important source of resveratrol, since the compound is also found in wine. In addition to its considerable economic value, grapevine (*Vitis vinifera*) and wine are now considered a key source of health-promoting secondary metabolites, especially antioxidant polyphenols such as resveratrol [23]. In red wine, it is found in relatively low quantities (0.3–7 mg/l aglycon form of resveratrol, and approximately 15 mg/l glycoside form) [24, 25].

The economic importance of grapevine has encouraged many researchers to study the physiological and molecular basis of berry development, particularly those processes affecting wine quality [26]. The availability of high-throughput analysis methods and high-quality information regarding the grapevine genome has led to the novel findings concerning the physiology of berry development at the transcriptome, proteome, and metabolome levels [27, 28].

In this species, a large array of STS genes, with 43 genes, identified and 20 of which shown to be expressed, thus suggesting the importance of stilbene metabolism for grapevine [29].

Resveratrol and Health Benefits: Effects *In Vivo* and *In Vitro*

Resveratrol possesses numerous important bioactivities including anti-inflammatory, antioxidant and antiaggregatory functions, and modulation of lipoprotein metabolism [30]. It has also been shown to possess chemopreventive properties against certain forms of cancer and cardiovascular disorders and to have positive effects on longevity [31–37]. Moreover, *trans*-resveratrol appears to protect against diabetes [38] and neurodegenerative disorders [39], due to induction of Sirtuin-1 genes [40]. *Trans*-resveratrol might also contribute to increasing the lifespan of metazoans and mice by miming the effect of caloric restriction and thus decreasing age-related signs [41, 42]. Experimental studies have shown that resveratrol exhibits both anti-inflammatory and cardioprotective potential by inhibiting the expression of inflammatory mediators and the monocyte adhesion to vascular endothelial cells [34, 35]. Considering the broad spectrum of protective properties, it has been proposed that resveratrol in red wine may explain the so-called "French Paradox" [43–46].

Resveratrol Bioavailability

Proof of the *in vivo* efficacy of resveratrol in man has been even less convincing. One of the main reasons for the *in vitro*/*in vivo* discrepancies may be the lack of information on resveratrol bioavailability *in vivo*, in particular following oral administration. Concentrations of resveratrol detected in tissue or at the cellular sites of action do not appear to be sufficiently adequate to demonstrate efficacy in man [47, 48].

The wide range of concentrations and doses used to achieve the various effects reported for resveratrol, both in *in vitro* cell culture and in animal studies, give raise to various questions regarding the concentrations that can be achieved *in vivo*. Furthermore, resveratrol has a short initial half-life and is considerably metabolized in the body. Nevertheless, the beneficial effects of resveratrol have been observed for thousands of years in the form of "*materia medica*", such as grapes and Ko-jo-kon, confirming the concept of the long-term effects of low levels of pharmacologically active substances. In this regard, it is important to better understand the molecular mechanisms of the long-term effects of resveratrol as far as concerns communication between functionally different cells.

Regarding the interaction of resveratrol with cell membranes, few studies have been published so far. It is believed, however, that this interaction may be involved in the biological activities of resveratrol since trans-membrane proteins are one of its cellular targets. Indeed, resveratrol is able to modulate the membrane organization which may consequently affect protein function. Therefore, the intracellular effects of resveratrol and the effects of this compound at membrane level were also revised since these aspects are essential for a better understanding of the pharmacological and therapeutic activities of this bioactive compound. Animal studies have been frequently used to investigate the bioavailability of phenolic compounds. In order to obtain information on absorption, metabolism, and the consequent bioavailability of resveratrol, various research approaches have been performed, including *in vitro*, *ex vivo*, and *in vivo* models. Most studies indicate that oral bioavailability of resveratrol is very low due to poor absorption and rapid and extensive metabolism and the consequent formation of various metabolites *i.e.*, resveratrol glucuronides and resveratrol sulfates [49].

Resveratrol Delivery

Despite the favorable therapeutic effects of resveratrol, the pharmacokinetic properties are not favorable since this compound has poor bioavailability, since it is rapidly and extensively metabolized and excreted. The therapeutic potential of resveratrol can only be adopted *in vivo* if the limitations related to its bioavailability can be overcome. Research programs are currently exploring other methods for enhancing resveratrol bioavailability, including: 1) co-administration with metabolism

inhibitors in order to prolong its presence *in vivo*, 2) the use of resveratrol analogous endowed with a better bioavailability, and 3) a drug delivery system employing nanotechnology [50–52]. As far as concerns the first approach, some authors have evaluated the possibility of enhancing the pharmacokinetic parameters of resveratrol by partially inhibiting its glucuronidation by means of co-administration with specific inhibitors [53].

Interest is also increasing in the second strategy, which consists in evaluation of new naturally-occurring and/or synthetic analogous of resveratrol endowed with the same structural backbone and some chemical modifications resulting in better efficacy [54]. In this context, the biological activity exerted by resveratrol metabolites is still debated. Therefore, further studies are needed, including the possibility to induce deglycosylation to release resveratrol into the target tissues [55]. Conventional formulations alone are probably inadequate to bring solutions to the physicochemical and pharmacokinetic limitations governing resveratrol bioavailability. To overcome this problem, novel drug delivery systems have been proposed to protect and stabilize resveratrol and to enhance its bioavailability. Nano- and micro-formulations for resveratrol encapsulation that include liposomes, polymeric nanoparticles, solid lipid nanoparticles, lipospheres, cyclodextrins, polymeric microspheres, yeast cell carriers and calcium or zinc pectinate beads, have been developed for resveratrol delivery [56]. The mode of drug delivery systems able to enhance resveratrol bioavailability is particularly promising. Research is currently focused on exploring multiparticulate forms in the millimeter to micrometer range and colloidal carriers in the nanometer range. As far as concerns route of administration research has focused on oral forms as these offer the possibility, if necessary, of easy chronic administration, but parenteral forms which avoid intestinal metabolism are also envisaged for acute treatment. For all current formulation attempts, the main issue is to determine whether the drug delivery system is efficient in improving the pharmacokinetic profile of resveratrol and promoting the *in vivo* therapeutic effects. Perspectives now lie in the development of innovative formulations able to overcome all the limitations governing resveratrol bioavailability as a pre-requisite for efficient and to assure efficient and sustained delivery *in vivo* [56].

Resveratrol Production in Transgenic Plants

Metabolic engineering has provided a method to improve not only polyphenol composition but also its levels. Promising results have been obtained with STS-encoding genes in transgenic plants, thus confirming that plant molecular engineering with resveratrol may lead to a novel functional food for human consumption.

STS genes have been transferred to a number of crops, either to improve the resistance of plant to stresses or the nutritional value (see Tables 1 and 2). The presence of resveratrol enhanced plant resistance to biotic and abiotic stresses such as fungal pathogens and UV radiation. Two stilbene synthase genes from *Vitis vinifera*, (Vst1 and Stsy) have been the most common genes used for plant transformation (Table 1). Other STS-encoding genes have also been used, notably the AhRS gene from *Arachis hypogea* [57, 58], the SbSTS1 gene from *Sorghum bicolor* [59, 60] and an STS-encoding gene from *Parthenocissus henryana* [61]. Furthermore, to increase the levels of stilbene production, some investigators have used chimeric genes or a combination of two STS encoding genes (Vst1 and Vst2*)* [62–65].

Modulation of gene expression is primarily controlled by the promoter selected to drive the transgene. To date, the STS-encoding genes for plant transformation have been expressed under the control of a limited number of promoters, in particular the well-characterized constitutive promoter pCaMV35S, its own stress-responsive promoter pVst1, the fungus-inducible promoter pPR10.1 or the tissue-specific promoter p-nap [66, 67, 86]. As expected, the pCaMV35S promoter triggered strong and constitutive stilbene accumulation in most studies, but, as a consequence, in some cases, caused a drastic depletion of the endogenous pools of precursors [58].

Qualitative and quantitative comparisons between the different transgenic plants synthesizing resveratrol and related stilbenes are difficult, as different analytical methods are used to assay these compounds. These analyses have shown various stilbene levels and spatiotemporal distributions, leading to a considerable variability in terms of relative amounts of different forms.

In particular, the glycosylation of polyphenolic compounds occurs frequently in plants not only to protect cells from their potential toxic effects but also to prevent their oxidation and enzymatic degradation [59]. In the case of resveratrol, the free compound is first synthesized before being glycosylated by endogenous glycosyl-transferases. Free resveratrol and its glycosylated forms have both been detected in transgenic plants [59]. The stilbene content also depends strongly upon plant species, probably on account of different endogenous pools of enzymes or precursors, as well as differences in secondary metabolic pathways.

As far as concerns the amounts of stilbenes detected in the various studies, no common trend has been found, which may possibly be explained by the plant species as well as the specific genetic constructs used for transformation. In tomato and apple, the free to glycosylated resveratrol ratio naturally depends on the fruit ripening stage [64, 81]. These two compounds accumulate differentially in fruit tissues at the mature stage [83]. These variations may be the result of different endogenous β-glycosidase expression patterns. Even more than the plant species considered, the stilbene levels and forms in transgenic plants depend not only on the

Table 1 Stilbene synthase gene expression in transgenic plants for resistance improvement

Plant species	Gene	μg/g	Biological activity	Reference
Nicotiana tabacum L.	*Vst1 and Vst2*	400	Resistance to *Botrytis cinerea*	[57]
	STS	50–200	Male sterility	[58]
Medicago sativa L.	*Arachis hypogea STS gene*	0.5–20	Resistance to *Phoma medicaginis*	[59]
Arabidopsis thaliana L.	*SbSTS1*	584	–	[60, 61]
Lactuca sativa L.	*Parthenocissus Henryana STS*	20–116	Resistance to *Fusarium oxysporum*	[62]
Solanum lycopersicum L.	*Vst1 and Vst2*	–	Resistance to *Phytophthora infestans*	[63]
Malus domestica Borkh	*Vst1*	–	–	[64]
	Vst1	3–7 without UV 23–62 under UV	–	[65]
Vitis vinifera L.	*Vst1*	–	*In vitro* resistance to *Botrytis cinerea*	[66]
	Vitis STS gene	2.6	–	[67]
Oryza sativa L.	*Vst1*	–	Resistance to *Pyricularia oryzae*	[68]
Hordeum vulgare L.	*Vst1*	–	Resistance to *Botrytis cineria*	[69]
Triticum aestivum L.	*Vst1*	–	Resistance to *Botrytis cineria*	[70]
	Chimeric STS gene	2	–	[71]
	Vst1 and Vst2	35–190	Resistance to *Puccinia recondita* and *Septoria nodorum*	[72]
Populus alba L.	*StSy*	309–615	No resistance to *Melampsora pulcherrima*	[73] [74]
Carica papaya L.	*Vst1*	54	Resistance to *Phytophtora palmivora*	[75]
Cavendish banana	*StSy*	–	*In vitro* tolerance to fungal diseases	[76]
Pisum sativum L.	*Vst1*	0.53–5.2	–	[77]
Actinidia deliciosa	*pSV25*	20–182	No resistance to *Botrytis cineria*	[78]
Rehmannia glutinosa Libosch	*AhRS3*	22–116 Up to 650	Resistance to *Fusarium oxysporum*	[79]
Fragaria x ananassa	*NS-Vitis3*	–	–	[80]

tissues or organs used as source material, but also on the fruit ripening stages [83].

In transgenic tomato, resveratrol synthesis was found to increase the overall antioxidant properties of the fruit, as well as the ascorbate/glutathione content [81] with a consequent two-fold increase in antioxidant activity of fruits. In another study, the hetherologous expression of STS in oilseed grape, combined with sinapate glucosyltransferase gene shutdown, considerably decreased the undesirable sinapate ester contents, thus improving the forage

Table 2 Stilbene synthase gene expression in transgenic plants for quality improvement

Plant species	Gene	μg/g	Biological activity	Reference
Solanum lycopersicum L.	*StSy*	53 (in fruit tissues)	Increased antioxidant capacity	[81]
	STS	30 (in fruit tissues)	Food quality improvement	[82]
	StSy	50–120 (in fruit tissues)	Modulation of other polyphenols	[83]
	StSy	10–120 (in fruit tissues)	Increased antioxidant and anti-inflammatory capacity	[84]
		50–180 (in flower tissues)	Increased antioxidant and anti-inflammatory capabilities, male sterility	[85]
Brassica napus L.	*Vst1*	361–616	Food quality improvement	[86]
Fragaria x ananassa	*NS-Vitis3*	–	–	[80]
Humulus lupulus L.	*Vst1*	490–560	Modulation of other polyphenols	[87]
Rehmania glutinosa Libosch	*AhRS3*	22–116 Up to 650 (under strees)	Antioxidant capacity	[79]

quality obtained from modified plants [86]. Taken together, these results suggest the overall relevance of STS-heterologous expression approach to obtain improved disease resistance and nutritional quality of agricultural crops and food products.

Functional Foods: Development and Investigations of the Effects of Resveratrol in Various Pathological Conditions

Metabolic engineering of plants will allow the development of foods with enhanced levels of various phytonutrients. It has been demonstrated for tomato plants where lines enhanced with flavonols have been developed by engineering the fruit-specific expression of a transcription factor that activates flavonol biosynthesis [88–90]. The improvement of resveratrol content in transgenic plants might benefit of this approach in the next future. Comparison of the effects of diets containing tomatoes enriched in different polyphenol classes upon onset and progression of diseases in cell assays or animal disease models will allow a quantitative assessment of the efficacy of different flavonoids within a common fruit matrix. One of the most intriguing results emerging from recent studies is that purified polyphenols, distributed as dietary supplements, do not have the same effects in promoting health as the same phytonutrients in a food context [91–93].

The nutritional context could influence the effects of polyphenols by affecting their bioavailability or be the result of various dietary phytonutrients acting synergistically, once absorbed [93, 94]. The impact of food on the efficacy of phytonutrients in promoting health could be assayed by comparing the effects of phytonutrients, to the same extent, in different foods in animal models [95–97]. Once functional food materials have been developed, the impact of target phytonutrients on a range of different chronic diseases can be assessed. For example, the pellets supplemented with resveratrol synthesizing tomato are currently being used in various disease models to assess the impact of transgenic tomato. In this way, studies on phytonutrients can evolve from models to humans using exactly the same experimental food matrix. The scientific information can then be used to program the development of new improved food products and the inclusion of these food products in dietary recommendations for specific disorders [97].

Conclusions

Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to improve the production of existing compounds, produce new compounds, or mediate the degradation of undesirable compounds. It involves the redirection of cellular activities by modifying the enzymes, endocellular localization, and regulatory functions within cells. Even more sophisticated metabolomic tools and analysis systems will offer the possibility to study both the primary and secondary metabolic pathways in an integrated fashion. Some interesting and important developments may be expected from plant transformation with STS gene. For example, promising results have been obtained with STS-encoding genes in transgenic plants, confirming that disease resistance can arise from foreign phytoalexin expression. In addition, plant molecular engineering with STS gene may lead to food products comprising edible legumes, cereals or fruits, which can be ingested, with their potential clinical benefits, by humans.

Taken together, these results suggest the general relevance of STS-encoding sequences as a tool for engineering disease resistance and the nutritional quality of agricultural crops and food products.

Therefore, further progress in a better understanding of the metabolic pathways and our ability to manipulate gene expression, in genetically modified plants, can be envisaged. The success of this approach depends upon the possibility to change the host metabolism and will depend primarily on a far more sophisticated knowledge of plant metabolism, especially the nuances of interconnected cellular networks.

Identifying rate-limiting steps in the synthesis of specific metabolites could provide targets for genetically engineering biochemical pathways to produce increased amounts of compounds as well as new compounds. Together with traditional plant breeding, genetic engineering provides great opportunities to develop plants with the desired levels and/or composition of specific polyphenols.

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