Phytostilbenes as agrochemicals: biosynthesis, bioactivity, metabolic engineering and biotechnology

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Abstract: Although constituting a limited chemical family, phytostilbenes represent an emblematic group of molecules among natural compounds. Ever since their discovery as antifungal compounds in plants and their ascribed role in human health and disease, phytostilbenes have never ceased to arouse interest for researchers, leading to a huge development of the literature in this field. Owing to this, the number of references to this class of compounds has reached the tens of thousands. The objective of this article is thus to offer an overview of the different aspects of these compounds

through a large bibliography analysis of more than 500 articles. All the aspects regarding phyto stilbenes will be covered including their chemistry and biochemistry, regulation of their biosynthesis, biological activities in plants, molecular engineering of stilbene pathways in plants and microbes as well as their biotechnological production by plant cell systems.

Keywords: Stilbenes, Phytoalexins, Plants, Biosynthesis, Biological activity, Metabolism, Engineering, Microbes, Bioproduction

1. Introduction

Throughout their life cycle, plants are challenged by numerous stresses, abiotic stresses on one hand, involving wounding, salt, drought, heat, colds, UV light and biotic stresses on the other, comprising attacks by numerous phytopathogenic microorganisms like fungi and bacteria as well as viruses and animals. To cope with these stresses, plants have evolved an intricate defence network including both constitutive,¹⁻³ and active resistance mechanisms.⁴⁻¹⁵ Among the arsenal of defences implemented by plants, phytoalexins, that is, secondary metabolite products of low molecular weight displaying biocidal activity, and produced by plants as a response to stress, have been the subject of an impressive number of studies during the last 30 years.¹²⁻¹⁵ Ever since the concept of phytoalexins was proposed by Müller and Börger in 1940,¹⁶ these compounds have indeed attracted considerable attention due to their role in plant-pathogen interactions. The seminal work of Langcake and Pryce has identified resveratrol, a stilbenic compound produced by grapevine, as a phytoalexin of that plant.¹⁷ This discovery yielded an intensive activity from this group between 1976 and 1981 also leading to the first characterization of resveratrol oligomeric forms.^{17,18} Since the work of Sieman and Creasy¹⁹ that was published a few years later established a relationship between the concentration of the phytoalexin resveratrol in wine and the beneficial effects of this compound on health, it is known that not only are phytoalexins implicated in plant defence mechanisms, but also many of them show interesting activities in human health and disease. The more spectacular results in this area have most certainly been obtained from the late 1990s onwards with phytostilbenes (hereafter named as stilbenes). In fact, convergent studies carried out in the 1990s on the characterization of resveratrol in wines on one hand,¹⁹ and the cardioprotective benefits of a moderate consumption of wine, the so-called French Paradox,²⁰ on the other, have highlighted the possible role of stilbenes in human health. A timely extension of these first observations was then given by the discovery of resveratrol's chemopreventive activity.²¹ Since then, stilbenes have proven to display several biological activities as anticancer,²²⁻²⁴ blood-pressure lowering agents²⁵ as well as neuroprotective compounds²⁶ conferring them a certain therapeutic potential.²⁷

Stilbenes are naturally-occurring polyphenolic compounds already found in various plant families including among others, Dipterocarpaceae, Pinaceae, Moraceae, Iridiaceae, Orchidaceae, Polygonaceae, Poaceae, Fabaceae, Gnetaceae, Cyperaceae, Aceraceae, Burseraceae, Euphorbiaceae, Meliaceae, Arecaceae, Paeoniaceae, Haemodoraceae, Vitaceae, Musaceae and Apiaceae which have previously been reviewed,^{28,29} with a particular emphasis on stilbenes from the Vitaceae.³⁰

Despite being the iconic representative of its group, resveratrol is but a simple constituent at the basis of a huge class of monomeric and oligomeric compounds,^{29,31} whose biosynthesis is catalyzed

by stilbene synthase (STS). Annotation of the grapevine genome has led to the identification of 43 to 48 *STS* genes revealing a large diversification of the genes putatively encoding for resveratrol biosynthesis and suggesting complex signaling and regulation pathways.^{32,33} Plant stilbenes have hitherto only been described as phytoalexins in a limited number of families,^{17,34,35} where they display antifungal activity against various plant pathogens.¹⁷ However, the action of a given phytoalexin cannot be envisaged unless taking into account the existence of a possible fungal metabolism of the latter³⁶ as well as mechanisms of phytoalexin insensitivity *via* transporters set up by fungi.^{37,38} Molecular engineering of the stilbene pathway in plants has been the subject of an intense research activity from the early 1990s leading to genetic transformations performed with *STS* genes from grapevine or from diverse plant origins in numerous hosts.³⁹⁻⁴¹ Finally, the main hindrance to the evaluation of the biological activity of stilbenes arises from the virtual impossibility to recover them in significant amounts using conventional chemical synthesis or plant extraction methods even though outstanding progress have been accomplished in the total synthesis of oligomeric stilbenes.⁴² Biotechnological processes, including metabolic engineering of stilbene pathways in microbes on one hand,⁴³⁻⁴⁵ and stilbene bioproduction in plant cell systems, on the other, constitute alternative and interesting approaches for stilbene sourcing.⁴⁶⁻⁵¹

All the aforesaid characteristics of stilbenes will thus be highlighted in the present review through a critical analysis of more than 500 articles, as well as the ongoing efforts that are under progress in the knowledge of this fascinating group of molecules. In section 2, we deliberately made the choice to present the stilbene chemistry based on the postulate that resveratrol constitutes the starting block of all stilbenes including monomeric and oligomeric structures rather than to present a “catalog” of all existing stilbenes, which has expertly been achieved in previous reviews.²⁹⁻³⁰ Section 3 constitutes a unique picture of all mechanisms taking part in the intricate regulation of stilbene biosynthetic pathways. We focus on stilbene antifungal activity in section 4 and show that resveratrol especially, due to its relatively low fungitoxicity, can be seen as a stress metabolite rather than as a phytoalexin. In section 5, a critical analysis of the gene and promoter options used for stilbene pathway engineering in plants is presented while we also discuss the benefits and limits of engineering stilbenes in plants in terms of disease resistance and functional food. Molecular engineering of stilbene pathways in microbial cells is presented in section 6 with a particular and critical emphasis on the techniques employed to improve stilbene titers in the recombinant microbial hosts. Finally, methods for the bioproduction of stilbenes by plant cell systems are discussed with regard to the diversity of the stilbenes produced, the yields obtained and their scalability for industrial purposes.

2. Stilbene chemistry and biochemistry

2.1. General aspects of stilbene biosynthesis

The biosynthetic route leading to stilbenes is a small branch of the phenylpropanoid route which can be considered as a parallel pathway of the flavonoid one. The first steps of the general phenylpropanoid pathway are common to most of higher plants. With the exclusion of grasses and some species of fungi and bacteria, which directly use tyrosine as a substrate, the synthesis of Coenzyme A (CoA)-activated forms of cinnamic acids starts from phenylalanine in the majority of

1 plant species. Stilbene monomers derive from the phenylalanine/polymalonate pathway as primarily
2 demonstrated by Langcake and Pryce.¹⁸ Phenylalanine ammonia lyase (PAL), which is the key enzyme
3 at the entry-point of the phenylpropanoid route, is considered to be more active than the tyrosine
4 ammonia lyase (TAL).⁵² Moreover, it is known that up to 30% of the carbon assimilated during
5 photosynthesis is channeled to the production of phenylalanine.⁵³ Simple stilbenes are classically
6 based on the 1,2-diphenylethylene skeleton constituted of two aromatic rings called A and B rings
7 respectively, and linked by an ethylene bond. Stilbene biosynthesis starts from D-glucose for the
8 building of the A ring or the B ring (Figures 1 and S1).

10 Insert Figure 1

12 Formation of the aromatic acids, phenylalanine and tyrosine ends with the the plastidial
13 arogenate pathway though recent evidence suggests a possible cytosolic production of phenylalanine
14 *via* the so-called prephenate route (Figure S1).⁵³ Phenylalanine, under the action of PAL, that is both
15 a constitutive and inducible enzyme, yields cinnamate, a fundamental precursor of all
16 phenylpropanoids including lignins, coumarins, and flavonols in plants, fungi, and bacteria.^{54,55}
17 Cinnamate can be either converted to *para*-coumarate (*p*-coumarate) by the cinnamate-4-
18 hydroxylase (C4H), a member of the ubiquitous cytochrome P450 hydroxylase superfamily (P450), or
19 directly transformed into its cinnamoyl-Coenzyme A (CoA) thioester through the action of the
20 cinnamoyl-CoA ligase (CL), ultimately affording pinosylvin (PS) (Figure 1). Alternatively, *p*-coumarate
21 may also originate from tyrosine by TAL. *Para*-coumarate is also converted to *p*-coumaroyl-CoA by
22 ligation to one CoA molecule under the action of 4-cinnamoyl-CoA ligases (4CL). The aforementioned
23 pathway called the phenylpropanoid pathway is at the basis of the building of the B-ring of stilbenes.
24 Condensation of either the *p*-coumaroyl-CoA or the cinnamoyl-CoA with three malonyl-CoA units
25 (constituting the A-stilbene ring) under the catalytic action of stilbene synthases (STS) (see below)
26 ends with the formation of simple stilbenes.⁵⁶⁻⁵⁹ Malonyl-CoA also derives from the glycolysis
27 pathway starting from D-glucose and yielding pyruvate and then acetyl-CoA by the pyruvate
28 dehydrogenase complex (Figure S1). Additionnally, malonyl-CoA can arise from the conversion of
29 malonate to malonyl-CoA upon the catalytic action of a malonyl-CoA synthase (*matB* gene).⁶⁰

30 Stilbene synthases belong to the polyketide synthases (PKS) of the type III family.^{59,61-64} Type III
31 PKS are mainly encountered in plants⁶⁴ as well as being found in fungi⁶⁵ and bacteria.⁶⁶ STS and
32 chalcone synthases (CHS) share similar characteristics, namely, they both are homodimeric proteins
33 of relatively low molecular mass (40-45 kDa) displaying a 75-90% identity among the 400 amino acids
34 which compose them and a highly conserved Cys-His-Asn sequence at the active site level.^{59,64} CHS
35 and STS possess the same scaffold for the recognition of substrate and the condensation reaction to
36 form the C6-C3-C6 skeleton of flavonoids with CHS and the C6-C2-C6 backbone of stilbenes with
37 STS.⁶⁷ Experimental evidence of the hypothesis that STS has evolved in some plants (for example,
38 pine, peanut and grapevine) in an independent manner from *CHS* genes, was provided by the
39 conversion of the alfalfa CHS through mutagenesis to a functional STS.⁵⁹ STS together with CHS,
40 represent the most studied enzymes of the plant type III PKS family, despite the existence of many
41 other type III PKS proteins. For this reason, this group is often referred as CHS/STS type III PKS family.

STS can be classified into two types: (a) *p*-coumaroyl-CoA specific type, such as the resveratrol synthase (EC 2.3.1.95) or (b) cinnamoyl-CoA-specific type such as the pynosylvin synthase (EC 2.3.1.146), depending on their preferential starting molecule. The former type mainly occurs in angiosperms such as peanut,^{35,68} grapevine,⁶⁹⁻⁷² sorghum,⁷³ mulberry,⁷⁴ and spruce,⁷⁵⁻⁷⁸ while the latter type is typically found in species belonging to the *Pinus* genus.^{79,80} A third category of STS could also be added to this subdivision, linked to the biosynthesis of piceatannol and capable of using caffeoyl-CoA as the starting substrate. To date, no enzyme having this biological function has yet been identified.^{75,81,82} Nevertheless, enzyme activity and kinetic analyses of recombinant STS from gymnosperms revealed that different STS, although exhibiting a preference for a given substrate, may even also accept different cinnamic acid derivatives and can be responsible for the production of various stilbenes depending on the starter molecule.⁸³

The first step in the synthesis of the polyketide intermediate involves the binding of the starter molecule *p*-coumaroyl-CoA to the enzyme CHS or STS. The CoA group found in each of the substrates (*p*-coumaroyl-CoA and malonyl-CoA) provides a common recognition feature for the active site of the enzyme. Once bound, an active site essential cysteine residue (Cys-164) reacts with the *p*-coumaroyl-CoA molecule, releasing CoA and leaving the *p*-coumaroyl group attached to the enzyme *via* a thioester linkage.⁸⁴ As aforementioned, PKS of type III use a great variety of Coenzyme A – tethered starting blocks though cinnamoyl-CoA and *p*-coumaroyl-CoA on one hand, and malonyl-CoA on the other, are the most frequent substrates of these enzymes. Both CHS and STS catalyzed sequential iterative decarboxylative reactions of *p*-coumaroyl-CoA with three malonyl-CoA units yielding respectively chalcones and stilbenes. Next, extension of the molecule begins with binding of the first malonyl-CoA molecule to STS. A catalytic histidine (or asparagine) in the STS active site catalyzes decarboxylation of the malonyl-CoA to give rise to the reactive acetyl-CoA anion. This reactive intermediate acts as a nucleophile to attack the thioester bond of the enzyme-linked *p*-coumaroyl group. This reaction extends the polyketide chain by one acetate unit, with the diketide (acetate coumaroyl) reattaching to the active site cysteine and releasing CoA. The entire process is repeated two more times, generating triketide and tetraketide chains. Both CHS and STS lead intermediately to a tetraketide following the condensation of the malonyl-CoA derived acetyl units. One molecule of CO₂ is lost at each condensation step.⁵⁹ The tetraketide intermediate formed as a CoA thioester evolves into two alternative ways according to different intramolecular cyclization mechanisms. For CHS, a C6 - C1 Claisen condensation affords naringenin-chalcone,⁵⁹ the first intermediate in the flavonoid pathway. For STS, the occurrence of a C2 – C7 aldol switch followed by the loss of one molecule of CO₂ leads to resveratrol. Since STS and CHS use the same substrates but perform different chemistry (aldol vs Claisen), this can explain the lack of flavonoid biosynthesis which has been reported in transgenic plants upon STS overexpression (section 5). In fact, *STS* gene overexpression seems to impede plant metabolism and particularly the flavonoid biosynthetic pathway. It has thus rapidly appeared that a competition between the two metabolic pathways, the flavonoid pathway controlled by chalcone synthase (CHS) on one hand, and the stilbene route controlled by STS on the other, could occur for precursor availability. Several works have clearly illustrated and discussed the fact that *STS* gene expression during plant engineering experiments (see section 5), can deprive CHS of its substrates resulting in the decrease of the level of some flavonoid

compounds such as rutin and naringenin in transgenic tomato,⁸⁵ the flavonol content in recombinant strawberry⁸⁶ or a 50% diminution of the anthocyanin content and a pale fawn color in *Arabidopsis*.⁸⁷

Otherwise, the number of malonyl-CoA units used upon the PKS-mediated cyclization process, appears to be not precisely controlled in STS as a pentaketide-CoA resulting from the condensation of four malonyl-CoA and yielding a new malonyl-resveratrol, has been described.⁶³ In fact, STS does not display a strict specificity regarding CoA starters as previous studies have reported that substrates such as methyl malonyl-CoA, myristoyl-CoA and propionyl-CoA can be used by this enzyme leading to unnatural stilbenes.⁸⁸

Simple stilbenes can display a great diversity in their chemical structures depending on the starting blocks used as substrates by STS, the number of malonyl-CoA units incorporated and the mode of cyclization of the final polyketides obtained. STS was indeed found to accept numerous CoA thioesters containing various heteroaromatic B rings such as thiophene, furan or fluorobenzene rings. For instance, the heterologous production of unnatural stilbenes has been reported with these substrates in a recombinant strain of *Escherichia coli* carrying a *STS* gene from *Arachis hypogea*.^{89,90} This also suggests that CoA ligases which operate upstream on the stilbene biosynthetic pathway are able to accept numerous precursors which structurally differ from their original substrates leading to various unnatural compounds such as fluorostilbenes, styrylthiophenes, styrylfurans and styrylpyridines.^{89,90} Numerous *STS* genes have been used to engineer microorganisms (yeast and bacteria) for stilbene production^{44, 91-93} as well as plants for disease resistance and functional food (sections 5 and 6).^{94,95}

2.2. Metabolism of stilbenes in planta: Action of “decorating” enzymes on the stilbene core

Various modifications can take place on the stilbene core under the catalytic action of peroxidases, which is at the basis of the polymerization process leading to resveratrol oligomeric structures as well as other reactions involving “decorating” enzymes (methyltransferases, prenyltransferases, hydroxylases and glucosyltransferases).

2.2.1. Prenylation of stilbenes

Although some flavonoid prenyltransferases have previously been characterized,^{96,97} the involvement of similar enzymes in stilbene chemistry has only been described recently^{98,99} despite the already old identification of isoprenylated forms of stilbenes in plants.^{100,101} Prenylation mainly consists of the transfer of a plastidial dimethylallyl pyrophosphate (DMAPP) group to resveratrol or piceatannol (3,3',4',5-tetrahydroxystilbene) to form respectively, arachidins 1-2 (3-methyl-2-butenyl-4-resveratrol or isopentenyl-resveratrol) and arachidin-3 (3-methyl-2-butenyl-4-piceatannol or isopentenyl-piceatannol) (Figure 2).⁹⁹ These latter compounds are prenylated in the 4-position, though prenylation may also occur at the 3'-position.^{102,103} Prenylated stilbenes have mainly been identified in peanut (*A. hypogea*).^{100,101} Using both transcriptomic and metabolomic studies, Medina-Bolivar's group was the first to characterize two genes from *A. hypogea* (*AhR3' DT-1* and *AhR4 DT-1*) encoding respectively the *A. hypogea* resveratrol 3'-dimethylallyl transferase and the *A. hypogea* resveratrol 4-dimethylallyl transferase.⁹⁹ These two enzymes located at the plastid level, specifically catalyze resveratrol prenylation on the 4-position (arachidin-2) and 3'-position (3-methyl-2-butenyl-

3'-resveratrol). The AhR4DT enzyme was indeed found to display a specific catalytic action regarding the transfer of a DMAPP unit at the 4-position of various hydroxystilbenes, resveratrol, piceatannol as well as pinosylvin.⁹⁷ These prenyltransferases thus work towards the diversification of stilbenes in peanut with positive effects on their biological activity and physico-chemical properties.

2.2.2. Methylation of stilbenes

Methylation is another frequent modification of plant phenylpropanoids, with hundreds of *O*-methylated products characterized so far, ranging from mono to polymethylated compounds belonging to the mono-lignol, chalcone, flavone, isoflavone, flavonol, anthocyanin and stilbene families.¹⁰⁴ This chemical modification is driven by *S*-Adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases (OMTs), which lead to the production of methylated stilbenes, such as pinosylvin 3-*O*-methyl ether in Scots pine¹⁰⁵ or pterostilbene in grapevine,¹⁰⁶ red sandalwood (*Pterocarpus santalinus*),¹⁰⁷ and blueberries (Figure 2).¹⁰⁸

Two resveratrol *O*-methyltransferase (*ROMT* or *SbOMT3*) genes were cloned from *Plasmopara*-infected grapevine leaves and from sorghum and their functional characterization indicated that they were able to catalyze resveratrol methylation to yield pterostilbene both *in vitro* and *in planta*.^{106,109} A lot of mono-methylated, di-methylated and even tetra-methylated stilbenes have already been identified in plants. Among them, the monomethylated stilbenes, pinosylvin 3-*O*-methylether from pine (*Pinus sylvestris*),¹¹⁰ isorhapontigenin (3'-*O*-methyl-piceatannol) and rhapontigenin (4'-*O*-methyl-piceatannol) from the petioles and roots of rhubarb (*Rheum rhaponticum*),¹¹¹ the dimethylated stilbene, pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) from grapevine¹¹² (Figure 2) and a tetramethylated one, combrestatin A4 (*cis*-3,5,4-trimethoxy-3-hydroxy-4'-methoxy stilbene) from *Combretum caffrum*,¹¹³ are the best known. Simple stilbenes thus undergo *O*-methylations which take place on their hydroxyphenyl groups. As for prenylation, stilbene methylation increases their biological activity as well as their membrane permeability.

O-methyltransferases from plants are organized in two groups.¹¹⁴ OMTs acting on stilbenes mainly belong to the type I OMTs group,¹¹⁵ most of them displaying some substrate specificity whereas some others are accepting multiple substrates and are thus defined as multifunctional enzymes. Type II OMTs seem to be rather involved in the *O*-methylation of cinnamoyl-CoA thioesters such as caffeoyl-CoA upon lignin biosynthesis.¹¹⁶ An OMT of type I designated PMT1 has long been described in pine (*Pinus sylvestris*) yielding pinosylvin 3-*O*-monomethylether from pinosylvin¹⁰⁵ though this enzyme did not display any substrate specificity for that stilbene. PMT1 indeed seems to show a higher affinity for methylation of piceatannol to yield isorhapontigenin (236% of relative activity compared to pinosylvin) but a lower one for resveratrol to form resveratrol 3-*O*-methylether (59% of relative activity).¹¹⁵ Surprisingly, the caffeoyl-CoA *O*-methyltransferase showed a non-negligible methylating activity on piceatannol. Recently, the *O*-methylation process of pinosylvin was revisited and a novel OMT named PMT2 was characterized in Scots pine (*P. sylvestris*) with decreasing affinity regarding the number of hydroxyphenyl groups: pinosylvin > resveratrol > piceatannol.¹¹⁵ PMT2 was found to be co-expressed with *STS* in Scots pine after wounding or a UV stress.

Insert Figure 2

The first report of pterostilbene as a phytoalexin from grapevine dates back to 1979.¹¹² Pterostilbene is a more potent antifungal compound against *B. cinerea*, the causal agent for gray mold as compared to resveratrol,¹¹⁷ and has a far lower aqueous solubility than its hydroxylated counterpart but seems, thanks to its higher lipophilic character, to be better absorbed at the gastrointestinal tract level.¹¹⁸⁻¹²¹ Langcake's group rapidly suggested that resveratrol could be the putative precursor of pterostilbene, however the characterization of a resveratrol *O*-methyltransferase (ROMT) only occurred later (see above).¹⁰⁶ Heterologous expression of other plant methyltransferases in recombinant plants¹⁰⁹ or microbial cells has confirmed their capabilities for stilbene methylation.¹²²⁻¹²⁴ Expression of the sorghum bicolor *O*-methyltransferase 3 (SbOMT3) in tobacco and Arabidopsis plants has revealed its specific methylating activity in the 3,5- position of resveratrol.¹⁰⁹ Simultaneously, the same authors have reported that a similar enzyme, SbOMT1 can catalyze single methylation of resveratrol at the 4'-position of the stilbene B-ring. Unexpectedly, specificity in the 3,5-dimethylating activity of SbOMT3, expressed in a recombinant *E. coli* strain, was not observed as only pinostilbene was recovered.¹²³ Expression of two resveratrol *O*-methyltransferases from *S. bicolor* (SbROMT1 and SbROMT2) in *E. coli* generated unnatural stilbenes instead of pterostilbene underlying the great diversification of methylation reactions taking place in stilbene chemistry (see sections 5 and 6).¹²⁴

2.2.3 Hydroxylation of stilbenes

Pinosylvin and resveratrol are respectively two polyhydroxystilbenes, a 3,5-dihydroxystilbene and a 3,5,4'-trihydroxystilbene. The piceatannol found in *Picea abies* displays a higher hydroxylation degree (3,5,3',5'-tetrahydroxystilbene).¹²⁵ Although the biosynthesis of pinosylvin (cinnamoyl-CoA + three malonyl-CoA units catalyzed by PS) and that of resveratrol (*p*-coumaroyl-CoA + three malonyl-CoA units catalyzed by STS) are well established, the origin of the fourth hydroxyphenyl group at the 3'-position of the B-ring of resveratrol is still unknown. Hammerbacher's group has assumed that piceatannol could arise 1) from the condensation of the appropriate cinnamoyl-CoA starter, caffeoyl-CoA with malonyl-CoA under STS action or 2) from the direct hydroxylation of the resveratrol backbone.⁷⁵ Nevertheless, caffeoyl-CoA was not proven to be a substrate for the STS of *P. abies* (genes *PaSTS1* and *PaSTS2*), a species which contains high amounts of this stilbene. Their results were rather in favor of a sequential process involving first the production of resveratrol which is then channeled to a 3'-hydroxylase analogous to the flavonoid 3'-hydroxylase (F3'H) implied in the biosynthesis of flavonoids and ensuring the direct hydroxylation of resveratrol.

Recombinant or not recombinant microorganisms and numerous microbial enzymes are able to catalyze non-specific hydroxylations of the stilbene core. For example, piceatannol has been produced in an engineered *E. coli* strain bearing two genes *HpaB* and *HpaC* encoding monooxygenases which display hydroxylation capabilities on stilbenes.¹²⁶ This compound was also obtained in a recombinant *E. coli* expressing a *p*-coumarate 3-hydroxylase gene from *Saccharothrix espanaensis*.¹²⁷ More recently, a highly regioselective hydroxylation of piceid (3-*O*-resveratrol- β -D-

glucoside) to yield astringin (3-*O*-piceatannol- β -D-glucoside) was reported with *CYP102A1* monooxygenase mutants from *Bacillus megaterium* (Figure 2).¹²⁸

2.2.4. Glucosylation of stilbenes

Glucosylation is a common modification of plant secondary metabolites which influences hydrophilicity, stability, subcellular localization and bioactivity of phenylpropanoid-derived compounds. Particularly, stilbene glucosylation could be involved in their solubility,¹²⁹ storage, transport from cytoplasm to apoplast, and protection from peroxidative enzymatic degradation.¹³⁰ There are several identified stilbene glucosides in Nature whose role is likely to increase the aqueous solubility of the poorly-hydrosoluble stilbene aglycones in biological compartments and fluids. The first report of a glucosylated form of resveratrol, piceid or polydatin (3-*O*-resveratrol- β -D-glucoside) was published in 1994.¹³¹ Since then, few other stilbene glucosides were characterized such as resveratrolside (4'-*O*-resveratrol- β -D-glucoside), astringin (3-*O*-piceatannol- β -D-glucoside) and isorhapontin (astralin-3'-*O*-methylether) (Figure 2). Little is known about the glucosyltransferases (GT) catalyzing the insertion of one or more glucosyl groups from UDP glucose (UDPG) on the stilbene core but this process appears relatively unspecific to stilbene compounds. A polyphenol glucosyltransferase with a potent activity on resveratrol glucosylation has been identified in *V. vinifera* cell cultures.¹³² De Luca's group reported few years later on the purification to almost homogeneity of a GT from the Concord variety (*Vitis labrusca*), which displays a dual functionality, the recombinant enzyme producing stilbene glucosides, on one hand, and glucoside esters of various hydroxycinnamic acids on the other.¹³³ The encoding gene for this enzyme named *VLRSGt*, was indeed found to share 92% identity to a gene encoding a *p*-hydroxybenzoic acid GT likely explaining its dual functionality. *VLRSGt* mainly yields the 3 and 4' glucosides, piceid and resveratrolside in grape berries.¹³³ Functional expression of GT from plants such as the *PaGT3* from *Phytolacca americana*¹³⁴ or the UDPGT gene *yjiC* from various *Bacillus* species¹³⁵ in recombinant *E. coli* strains also resulted in the production of piceid and resveratrolside. Here again, no regioselectivity was observed during the glucosylation reactions of stilbene compounds.

2.3. Oligomeric resveratrol-based structures: Resveratrol, the building block of all stilbenes?

2.3.1. Dimeric structures

In their outstanding review on stilbene chemistry, Stephenson's group has clearly demonstrated that most of the stilbene dimers identified to date, originate from resveratrol.²⁹ Primitively, the entire polymerization process seems to begin by the oxidation of single resveratrol units by peroxidases as suggested by the group of Langcake in their seminal work.¹³⁶ The loss of one electron and one proton in resveratrol leads to one starting phenoxyl radical. Delocalization of the charge on the aromatic structure then allows the formation of various mesomeric forms termed as A, B, C, D and E, and whose condensation yields to the building of the main resveratrol dimers known to date (Figure 3).

Oxidation of resveratrol is obtained under the catalytic action of peroxidases. This process appears to be unspecific as, together with grapevine peroxidases, numerous other peroxidases are also involved. It was first demonstrated that resveratrol could be prone to the action of the

horseradish peroxidase (HRP) *in vitro* in the presence of H₂O₂ leading to δ -viniferin, a grape phytoalexin mimic.¹³⁶ Beside HRP which is the most commonly used peroxidase enzyme for the *in vitro* dimerization of stilbenes,¹³⁷⁻¹⁴⁰ other peroxidases such as the peroxidases from soybean (*Glycine max*), *Arthromyces ramosus* and *Momordica charantia* have also been employed.^{137,141,142}

Insert Figure 3

In planta, the resveratrol oxidative coupling to form oligomeric compounds seems to be controlled by peroxidases which have particularly been studied in grapevine by the group of Calderon in the 90's. Three groups of isoenzyme peroxidases were characterized as peroxidases A1 and B2 mainly localized in the cell wall and the apoplastic compartment as well as vacuolar peroxidases (B5 group).¹⁴³⁻¹⁴⁵ The presence of peroxidases at the cell wall level or in the apoplast which are involved in the plant extracellular proteome,¹⁴⁶ is in agreement with the recovery of large amounts of stilbene dimers in the extracellular medium of grapevine cell suspensions indicating a polymerization of resveratrol units taking place at this level.^{51,147,148} From both a biochemical and a physiological point of view, the production of resveratrol oligomers could be seen as a spectacular illustration of the capacity of plants to synthesize complex chemical structures on one hand, and the ability of the plant cell to diversify its arsenal of defenses through the accumulation of various antifungal compounds, on the other. Unexpectedly, it was shown that fungal cells or fungal laccases extracted from *B. cinerea* filtrates were also able to dimerize stilbene monomers as a catabolic action to counteract phytoalexin antifungal activity (see section 4).^{36,149-151}

Stephenson's group has identified four main coupling modes of the resveratrol phenoxyl radicals which may account for the best-known resveratrol dimers.²⁹ These different modes of coupling were named 3-8', 8-8', 8-10' and 8-12' according to the numbering of the respective carbons involved in resveratrol unit condensation as illustrated in Figure 3. The most characteristic dimer resulting from the 3-8' coupling of resveratrol phenoxyl radicals, is δ -viniferin also designed as the resveratrol dehydrodimer. δ -Viniferin is a resveratrol dimer structurally very close to the natural phytoalexin, ϵ -viniferin, which is formed according to a 8-10' coupling.^{36,136} As aforementioned, δ -viniferin was recovered as an unexpected result of enzymatic trials to obtain ϵ -viniferin using HRP with H₂O₂ in a water/acetone mixture,¹³⁶ or as a catabolism product of the fungal laccase-mediated activity on resveratrol.^{36,151} δ -Viniferin was also reported as a major component of the grapevine defense response to an attack by powdery mildew (*P. viticola*).¹⁵²

Some examples of the putative process leading to 8-8' dimers are displayed in Figure 4. The starting point of the formation of these dimers is the 8-8' coupling of the same two hydroxyphenyl radicals C resulting in two hypothetical intermediates. As reported by Stephenson's group,²⁹ the first hypothetical intermediate presents an interesting symmetry of the two *para*-quinone methide groups carried by the two carbons, C8a and C8b. From that point on, the bicyclic skeleton of ($\pm$)-pallidol is obtained following two successive Friedel-Crafts cyclizations (C10a and C8b; C10b and C8a) as allowed by the appropriate configuration of the substituents around the C8a-C8b bond.

Water addition to this first 8-8' dimer intermediate affords restrytol A (Figure 4), a resveratrol metabolite resulting from the laccase-mediated resveratrol dimerization¹⁵¹ or

tricuspidatol A, a resveratrol dimer from *Parthenocissus tricuspidata* (not pictured).¹⁵³ The second hypothetical intermediate undergoes one Friedel-Crafts cyclization (C10b and 8a) but the *anti,anti*-configuration of the resulting *para*-quinone intermediate is unfavorable to a second Friedel-Crafts cyclization.²⁹ From that point on, water addition affords leachianol while a tautomerization process yields to the indane-derived stilbene from *Parthenocissus quadrangularis*, (-)-quadrangularin A (Figure 4).¹⁵⁴ (+)-Leachianol F/G and (-)-quadrangularin A have been extracted from *V. vinifera* stalks and reported to display negative effects against the HIV-integrase.¹⁵⁵

Insert Figure 4

The condensation of resveratrol hydroxyphenoxy radicals according to the 8-10' coupling mode leads to well-known stilbene dimers such as ϵ -viniferin and some components of the ampelopsin group (ampelopsins B, D and F) (Figure 5). The first resveratrol dimer identified as a phytoalexin from the Vitaceae was the (+)- ϵ -viniferin,^{18,156} while the occurrence of its enantiomer (-)- ϵ -viniferin has been reported in other plant families, Dipterocarpaceae,¹⁵⁷ Cyperaceae,¹⁵⁸ and Gnetaceae.¹⁵⁹ (+)- ϵ -Viniferin from grapevine is formed upon the coupling of the two radicals D and C leading to an intermediate *para*-quinone (Figure 5).²⁹ The characteristic dihydrobenzofuran ring of viniferin is formed through an oxa-Michael addition (path B, Figure 5). The 8-10' dibenzocycloheptane, (+)-ampelopsin B (Iridiaceae)¹⁶⁰ is then putatively obtained from (+)- ϵ -viniferin. A Friedel-Crafts reaction between carbons 8a and 7b can also take place in the intermediate *para*-quinone (path A) (Figure 5), followed by a second Friedel-Crafts cyclization (carbons 10b and 7a) leading to the dibenzobicyclo[3.2.1]octadiene dimer, (+)-ampelopsin F isolated from *Ampelopsis brevipedunculata*.¹⁶¹ On the other hand, tautomerization yields (-)-ampelopsin D.¹⁶²

Insert Figure 5

Some oxidized 8-10' dimers (ampelopsin A, balanocarpol, malibatol or hopeahainanphenol) originate from (-)- ϵ -viniferin. Opening of the putative epoxide of (-)- ϵ -viniferin (Figure 6) affords a second intermediate which undergoes a Friedel-Crafts cyclization yielding the 8-10' dibenzocycloheptane dimer from the Dipterocarpaceae, (+)-balanocarpol¹⁶³ and its epimer, (-)-ampelopsin A identified in *A. brevipedunculata* (Vitaceae) (not pictured).¹⁶¹ The oxidation of balanocarpol then yields a hypothetical intermediate which can lose one molecule of water leading to (-)-malibatol A, a stilbene dimer from *Hopea hainanensis*,¹⁶⁴ or undergo an oxidative dearomatization process to form hopeahainanphenol (Figure 6).¹⁶⁵

The putative relationships between other 8-8' dimeric naturally-occurring stilbenes are relatively easy to deduce (Figure S2). (-)-Parthenocissin A, a dimer from *Parthenocissus quinquefolia*¹⁶⁶ results from the simple photoisomerization of the *trans*-stilbene moiety of (-)-quadrangularin A. Then an oxidative process leads to the phenanthrenic ring of (+)-laetevirenol A, a dimer from *Parthenocissus laetevirens*.¹³⁸ On the other hand, vitisinol A (*A. brevipedunculata*)¹⁶⁷ could arise from restrytol A through a double process including dehydrogenation and loss of one molecule of water (Figure S2). This proposed mechanism is purely hypothetical as restrytol is a

resveratrol catabolite dimer produced by the laccase of *B. cinerea*¹⁵¹ while vitisinol is a dimer isolated from the Vitaceae.

Insert Figure 6

2.3.2. Highly condensed stilbene oligomers

Trimers, tetramers as well as more complex resveratrol oligomers such as heptamers, are supposed to follow a similar building scheme including the same modes of coupling (8-10', 10-8', 3-8' and 8-8'). These mechanisms were also deeply deciphered by Stephenson's group.²⁹ For example, the two trimers, gnetin H and miyabenol C are obtained from the 10-8' and 8-10' coupling of oxidized forms of (-)- ϵ -viniferin with the resveratrol hydroxyphenyl radicals C and D, respectively. Both trimers display a supplementary dihydrobenzofuran ring formed through an oxa-Michael addition from their corresponding hypothetical intermediates (Figure 7). These hypothetical mechanisms are confirmed by the results of He's group¹³⁸ which clearly showed that various resveratrol trimers such as parthenocissin B and laetevirenols A-E are obtained in 14-15% yields through the simple condensation of resveratrol with various starting dimers using HRP.

Insert Figure 7

Tetramers such as ampelopsin H, viniferol A, (-)-hopeaphenol (Dipterocarpaceae) and (+)-hopeaphenol (Vitaceae) are supposed to result from the 8-8' coupling of two oxidized molecules of (-)- ϵ -viniferin (Figure 8). The same hypothetical intermediate is obtained from which the three tetramers are generated through a symmetric Friedel-Crafts cyclization reaction involving carbons 14b and 7c as well as carbons 14c and 7b for ampelopsin H, which is a stilbene tetramer from *A. brevipedunculata*.¹⁶¹ Viniferol A, a stilbene tetramer from *V. vinifera* stems,¹⁶⁸ is obtained from a cyclization involving carbons 14b and 7c, carbons 10d and 7b. Hopeaphenol results from Friedel-Crafts reactions between carbons 10d and 7c, carbons 10a and 8b, respectively (Figure 8). The two tetramers, vitisin A and vitisin B isolated from grapevine root extracts,¹⁶⁹ result from the 3-8' coupling of two oxidized molecules of (-)- ϵ -viniferin yielding a hypothetical intermediate (Figure S3). From that point on, vitisin B is formed via an oxa-Michael addition (path A) while the dibenzocycloheptane tetramer vitisin A is obtained by a Friedel-Crafts cyclization (path B).²⁹

Insert Figure 8

As an illustration of the hypothesis that highly condensed resveratrol oligomers can be seen as the result of the addition of resveratrol oligomers of a lower size, the putative origin of the resveratrol heptamer pauciflorol D identified in *Vatica pauciflora*¹⁷⁰ was deciphered by Stephenson's group.²⁹ It was suggested that a Friedel-Crafts reaction (carbons 12c and 7e) takes place between the resveratrol 8-10' tetramer, vaticaphenol A, a stilbene tetramer from *Hopea acuminata*¹⁷¹ and an 8-8' quinone trimer formed by the coupling of three resveratrol hydroxyphenyl radicals C (Figures 3 and S4). Vaticaphenol A likely comes from the 8-10' coupling of two oxidized molecules of (-)- ϵ -viniferin.

It is evident from the above-mentioned elements that the oxidative coupling of the resveratrol phenoxyl radicals upon stilbene oligomerization occurs with regioselectivity. Data regarding the enantiomeric purity of the obtained products are often not available as determination of their absolute configuration is sometimes lacking, and mainly concerns stilbene dimers.²⁹ A few dimers have been isolated as racemic compounds such as the ($\pm$)-pallidol ($[\alpha]_D = 0^\circ$) but most of them are found as single enantiomers. For example, ϵ -viniferin exists in both enantiomeric forms depending on the considered plant families. The (-)- ϵ -viniferin occurs in the Gnetaceae, Fabaceae and Dipterocarpaceae families while the other enantiomer, (+)- ϵ -viniferin, is only found in the Vitaceae. (-)-Ampelopsin is present in the Vitaceae with the (+) enantiomeric form being found in the Fabaceae, etc. The occurrence of many natural stilbene oligomers as optically active compounds and rarely as racemics thus strongly supports the hypothesis of an enantioselective coupling of the phenoxyl radicals formed upon oligomerization process implicating chiral entities.²⁹ In fact, a question that still remains is the deciphering of the mechanisms which create stereoconditions controlling the genesis of optically active stilbenes.²⁹ It has been suggested that putative dirigent proteins may be involved in the regio and stereoselective coupling of phenols as already reported by Pickel et al.¹⁷² For example, an oxidative process similar to that described for stilbene oligomerization (Figure 3), occurs in the dimerization of the monolignol units upon lignan biosynthesis in *Forsythia intermedia*. During this process, dirigent proteins (DIRs) are able to orientate the laccase-catalyzed coupling of the coniferyl alcohol radicals. Specificity in the coupling orientation was found to be linked to the nature of the DIRs. Functional characterization of one DIR from *Arabidopsis thaliana* AtDIR6 led to the enantioselective coupling of coniferyl alcohol to (-)-pinoresinol while a DIR from *F. intermedia* FiDIR1 oriented the synthesis to (+)-pinoresinol, with the racemic ($\pm$)-pinoresinol being formed in the absence of DIR.¹⁷² However, to our knowledge, such proteins remain to be characterized in stilbene chemistry.

Although we have limited this presentation to a few characteristic examples, one can understand that the chemistry of stilbenes is extremely complex. What is still surprising is that this extraordinary diversity seems to be based on a single molecule, resveratrol. For a very complete vision of this subject, we refer the reader to the already mentioned review by Stephenson's group.²⁹

3. Stilbene synthases and stilbene biosynthesis regulation

3.1. Stilbene synthase (STS): evolution from chalcone synthase and gene family organization

The STS protein was firstly extracted and purified in 1984 from stressed cell suspension cultures of peanut (*Arachis hypogea*).⁵⁸ The enzyme was found to be a dimer of estimated molecular weight 90 kDa with an iso-electric point (pI) of 4.8. CHS and STS are proteins of approximately 390-400 amino-acid residues, depending on the species. A conserved cysteine residue located in the central section of these proteins has been shown to be essential for the catalytic activity of both STS and CHS enzymes and represents the binding site for the *p*-coumaroyl-CoA starting substrate.¹⁷³ The region around this active site is well conserved and can be used as a signature pattern for CHS and STS. This shared domain, indicated in PROSITE (<http://expasy.org/prosite>)¹⁷⁴ as the CHS/STS active site has the following pattern (PS00441): R-[LIVMFYS]-x-[LIVM]-x-[QHG]-x-G-C-[FYNA]-[GAPV]-G-[GAC]-[STAVK]-x-[LIVMF]-[RAL]. The two proteins show a high degree of similarity based on sequence

homology, which reaches approximately 75% depending on the species, the gene structure and by comparison of their crystallographic structures.¹⁷⁵ Gene structure is also conserved between members of the two gene families, with a single intron exactly at the same position, *i.e.* within the triplet coding for Cys-60.⁵⁷

Phylogenetic analyses of STS and CHS families indicated that STSs of Scots pine, peanut and grapevine do not form a separate cluster but instead a cluster with the CHS proteins from the same or related plants.⁵⁷ This observation suggests there was not an ancestral *STS* gene in the strict sense and that STS evolved from CHS several times in the course of evolution.¹⁷⁶ This hypothesis was reinforced by the observation that only three amino acids exchanges were required within the N-terminal 107 aa of a CHS to STS function in a chimeric protein obtained from a fusion of CHS and STS protein fragments.

To date, *STS* genes have been identified in peanut, spruce, pine, mulberry, grapevine and sorghum,^{177,178-182} which is the only monocotyledon in which an *STS* has been found. Apart from sorghum, for which only one *STS* member has been identified,¹⁷⁷ *STS* genes are organized in small families composed of 1-10 members in all other plant species. For example, ten *STS* genes have recently been identified in the genome of mulberry tree (*MnSTS1-10*),¹⁸² two paralogues were identified in peanut.⁵⁷ Within the *Pinus* genus, at least five pinosylvin synthase genes were cloned in Scots pine (*PST1-5*),¹⁷⁸ three members were detected in Japanese red pine (*PdSTS1-3*),⁸³ and two in Eastern white pine.¹⁸³ The only exception in *STS* family size is grapevine (*Vitis vinifera* L.), which counts up to 48 members of which at least 33 are potentially functional as revealed by the genome-wide analysis of the grapevine genome.^{33,184} Evolutionary and functional analysis of the grapevine *STS* gene family revealed that its evolution was dominated by purifying selection, with no evidence for strong selection for new functions among *STS* genes. As a matter of fact, functional characterization of nine paralogues representing most of the grapevine *STS* gene family diversity clearly indicated that these genes do encode for proteins with STS activity. There is no clear evidence for a functional diversification of *STS* genes and a lack of experimental data indicating whether different members of grapevine and other plant *STS* multigene families vary in their enzyme activities and specificity for stilbene biosynthesis. However, expression analyses in grapevine and mulberry suggested a transcription sub-functionalization of paralogues, *STS* genes exhibiting differences in expression between organs and tissues, during development, as well as in response to environmental stresses.^{33,185,186}

3.2. Stilbenes as constitutive and stress responsive compounds

The accumulation of stilbenes, from both a qualitative and quantitative point of view, has most intensively been studied for species belonging to seven main families: *Vitaceae* (grapevine), *Pinaceae* (pine and spruce), *Poaceae* (sorghum, sugarcane, barley, and fescue), *Ericaceae* (blueberry and deerberry), *Fabaceae* (peanut and false acacia), *Moraceae* (mulberry), and *Polygonaceae* (knotweed and rhubarb) (for a detailed review see ¹⁸⁷). Several plant species including pine (*Pinus* spp.), spruce (*Picea* spp.), knotweed (*Polygonum cuspidatum*), gnetum (*Gnetum parvifolium*), mulberry (*Morus alba*), and rose myrtle (*Rhodomyrtus tomentosa*) constitutively produce large amounts of stilbenes in natural unstressed conditions. Depending on the species considered, these

compounds are accumulated in different organs and tissues, including leaves, roots, fruits, seeds, stalks, wood, bark, or needles. In pine as in other trees such as *Eucalyptus* (*Eucalyptus regnans*) and *Maclura* (*Maclura pomifera*), stilbenes occur primarily in the dead or moribund tissues of the heartwood and bark, while they seem to be scarcely constitutively produced in green tissues like leaves and sapwood.^{188,189} Conversely, in other species such as rose myrtle or mulberry, they are also accumulated in green tissues, although at lower levels.¹⁹⁰ Several studies reported on resveratrol and piceid quantification in commercial edible fruits and vegetables, including strawberry, raspberry, cherry, pepper, tomato, cucumber, carrot, eggplant, lettuce, plum, apple, pear, peach, and grape as well.^{191,192} Among these, grape is the fruit containing the highest amounts of stilbenes. As a matter of fact, although most of the studies on *Vitis* spp. were focused on the role of stilbenes as inducible compounds, it was demonstrated that grapevine berries constitutively accumulate stilbenes at the skin level during the ripening process.^{69,193} Not only the amount of produced stilbenes seems to be genotype-dependent with “high” stilbene producers such as Pinot noir and “low” stilbene producers like Corvina,^{194,195} but also extremely variegated: recent qualitative and quantitative studies on the profiling of grape stilbene derivatives performed using modern approaches, revealed that a total of 18-21 stilbenes are accumulated in *V. vinifera* berries.^{71,196} Moreover, based on Wang et al.,¹⁹⁷ young grapevine plants are able to accumulate resveratrol also in the stem (xylem and phloem), axillary buds, shoot tips, petioles, roots, and leaves.

Despite the above-mentioned evidences, the majority of studies on the induced biosynthesis and accumulation of stilbenes have indicated that these compounds are moderately expressed under normal conditions but strongly accumulate in response to a wide range of biotic and abiotic stresses, as a result of an increased transcription of *STS* genes and the coordinate activation of upstream genes belonging to the general phenylpropanoid pathway, such as *PAL* and *C4H* (Figure 1). Among the abiotic stresses inducing stilbene biosynthesis are wounding or mechanical damage,^{33,79,198-202} UV-C light irradiation,^{69,72,197,203-206} treatments with chemicals such as aluminum ions, cyclodextrins (CD),²⁰⁷⁻²⁰⁹ ozone,^{207,210-212} exposure to high light or high temperature,^{206,213,214} and drought, depending on the species and the genotype.^{70,215-217} The synthesis of stilbenes as a response to biotic stresses, is particularly well documented in grapevine where it has been shown to be induced upon infection with different fungal pathogens, including powdery mildew (*Erysiphe necator*),^{218,219} downy mildew (*Plasmopara viticola*)¹⁵⁶ and grey mould (*Botrytis cinerea*).^{220,221}

3.3. Signaling pathways regulating stilbene biosynthesis

3.3.1. Regulation by calcium signaling

Calcium represents an important activator of plant defense responses to both abiotic and abiotic stresses,^{222,223} and it was demonstrated to be involved in the regulation of stilbene biosynthesis in grapevine. It was shown that the treatment of grapevine (*V. vinifera* cv. Gamay) cell suspensions with BcPG1, an endopolygalacturonase from *B. cinerea*, which acts as a potent elicitor of defense responses in grapevine, is able to trigger a Ca^{2+} influx within a few minutes. This increase in Ca^{2+} induces the phosphorylation-dependent production of nitric oxide (NO), which in turn is involved in a number of processes including regulation of cytosolic calcium homeostasis by activating Ca^{2+} release from internal stores and regulating Ca^{2+} fluxes across the plasma membrane, variation of

plasma membrane potential, production of active oxygen species (AOS) and enhanced expression of defense genes, including *PAL* and *STS* and increased stilbene production levels.^{224,225} Subsequent studies based on the use of Ca^{2+} ionophores or channel blockers supported these evidences, demonstrating that increase in the cytosolic Ca^{2+} concentration is fundamental for the MeJA- and cyclodextrin-mediated induction of stilbene biosynthesis.²²⁶⁻²²⁹ Recently, Luo et al.²³⁰ showed that treatment with a Ca^{2+} ionophore induces the expression of a transcription factor (TF) involved in the regulation of *STS* in *V. quinquangularis*-Pingyi and can be associated to early signaling events in the salicylic acid (SA)-mediated flg2-triggered plant immunity. Kiselev et al.²²⁸ showed that, together with *STS* genes, treatment of grapevine cell cultures with Ca^{2+} channel blockers and a Ca^{2+} ionophore also affected the mRNA levels of calcium-dependent protein kinase (*CDPK*) genes. CDPKs are known as essential Ca^{2+} sensors in plant cells regulating plant development and responses to environmental stress²³¹ and application of inhibitors of their activity considerably reduces resveratrol accumulation and expression of *STS* genes in grapevine cell cultures with the (SA)- and precursor-induced stilbene accumulation.²³²⁻²³⁵ The role of two CDPKs, namely CPK20 and CPK29, in the regulation of stilbene biosynthesis was confirmed by overexpressing these genes in grapevine cell cultures and by resveratrol quantification.^{203,236} These results indicated that some CDPKs are positive regulators of stilbene biosynthesis.

3.3.2. Regulation by reactive oxygen species

Reactive oxygen species (ROS) are produced and accumulated as a result of a perturbed redox balance in response to various biotic and abiotic stresses. Recent studies have indicated that ROS not only act as toxic compounds that damage cells, but also as pivotal early-signaling molecules.²³⁷⁻²³⁹ There is accumulating evidence demonstrating that ROS are involved, probably as intermediates, in the signaling pathways regulating stilbene biosynthesis in different plant species. In peanut, treatment with H_2O_2 and paraquat, a ROS generator, induces *STS* expression and resveratrol biosynthesis.²⁴⁰ Faurie et al.²²⁷ and Belchí-Navarro et al.²²⁹ demonstrated that the methyljasmonate (MeJA)- and cyclodextrin (CD)-induced biosynthesis of stilbenes in grapevine cell suspensions is dependent on the production of O_2^- and H_2O_2 (see also section 7). The accumulation of ROS seems to be a necessary but not sufficient condition for *STS* induction and stilbene accumulation in cell lines of cultivated and wildtype grapevine (*V. vinifera* cv. Pinot Noir and *V. rupestris*) treated with hairpin, a bacterial effector.²⁴¹ Treatment of detached grapevine berries with menadione, an oxidative stress inducer releasing O_2^- , H_2O_2 , and $\text{OH}^\cdot$, increases stilbene production both at pre-*véraison* and *véraison* stages.²¹⁴ As above mentioned, in addition to ROS, the subgroup of reactive nitrogen species (RNS), including NO, is also involved in the regulation of stilbene biosynthesis as mediators in the Ca^{2+} dependent production of AOS and *STS* induction.^{225,229} More recently, Bai et al.²⁴² have proposed a model where stilbene accumulation in the cultivar *V. labrusca* Concord following Al^{3+} and UV-C treatments involves ROS production as an early signal.

3.3.3. Regulation by hormone-related signaling pathways

A conspicuous number of studies have concerned the involvement of plant hormones in the regulation of stilbene biosynthesis. Amongst these are jasmonic acid (JA), its methyl ester, methyl jasmonate (MeJA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA).

Jasmonates constitute a group of oxygenated fatty acid derivatives produced *via* the oxidative metabolism of polyunsaturated fatty acids that are implicated in many processes including the regulation of plant defense responses.²⁴³ The role of JA and its derivatives in the regulation of stilbene biosynthesis has been described in many studies. In grapevine, treatment with JA or MeJA has been associated to an increase in stilbene accumulation both in cultivated varieties and in cell cultures.^{214,215,227,244-247} (see also section 7).

Salicylic acid is another plant hormone involved in stress responses. In particular, SA is known to act as a mediator in plant local and systemic responses to infection with plant microbial pathogens and abiotic stresses.²⁴⁸ The role of SA in stilbene biosynthesis is still unclear, since several studies have shown that treatments with this hormone are associated to an accumulation of resveratrol or other stilbenes in cell cultures of both cultivated and wild grapevine species or *Polygonum*,^{245,249} but some others indicated that this hormone can interfere with other phytohormones such as ET or MeJA reducing their positive effect on resveratrol production.²²⁹ Recently, it was shown that SA activates the promoter of *VqMYB14*, a transcription factor (TF) involved in the regulation of stilbene biosynthesis in *V. quinquangularis*-Pingyi, but had no such effect on the promoter of the orthologous TF in *V. vinifera* (*VvMYB14*).²³⁰ Thus, it could be possible that SA regulates stilbene biosynthesis in grapevine in a species- and variety-dependent manner.

Ethylene is a gaseous hormone involved in many different plant physiological and developmental processes including senescence, fruit ripening, abscission and stress response.²⁵⁰ Although ET is not considered an inductor of plant secondary metabolism, several studies have indicated that treatments with this hormone or with ET-releasing compounds such as ethephon, are associated to an accumulation of stilbenes and an increased expression of their biosynthetic genes. This was observed in peanut,²⁴⁰ grapes,²⁵¹ and grapevine leaves.²⁵² Although not representing a direct evidence for the involvement of ET in the regulation of stilbene biosynthesis, Vannozzi et al.²⁰², in a large-scale co-expression analysis based on public grapevine expression data, identified several TFs belonging to the AP2/ERF family that are strongly co-expressed with *STS* genes. Moreover, recent surveys showed that various AP2/ERF TF-binding sites (TFBSs) are present within the 1 kb promoter region of many grapevine *STS* promoters.²⁵³ All these evidences suggest that ET is involved in the signaling pathways modulating the production of stilbenes in plant cells.

Finally, a few studies have also related abscisic acid (ABA) to the increased accumulation of stilbenes. Recently, it was demonstrated that the exogenous application of ABA to detached grapevine berries leads to a progressive increase in the content of resveratrol, piceid, and viniferin in pre-*véraison* berries.²¹⁴ Nicolas et al.²⁵⁴ showed that the overexpression of a gene encoding for a grapevine ABA-responsive basic leucine zipper transcription factor, namely *VvABF2*, strongly promotes the accumulation of *trans*-resveratrol and *trans*-piceid in transgenic grapevine cell lines, especially after treatment with ABA. More recently, Wang et al.²⁵⁵ isolated a basic leucine zipper (bZIP) transcription factor, *VqbZIP1*, from Chinese wild *V. quinquangularis*, which is involved in the

ABA signaling pathway and regulates stilbene synthesis. The effect of ABA on stilbene biosynthesis was also observed in other plant species such as *Marchantia polymorpha*,²⁵⁶ where ABA seems to induce biosynthesis of *bis*-bibenzyl compounds, a group of stilbenoids found in the liverwort.

Taken together, all these evidences suggest that the phytohormones JA, MeJA, SA, ET, and ABA are positively involved in the regulation of stilbene biosynthesis. Conversely, no data are available about the role of other plant hormones such as gibberellins (GA) and brassinosteroids (BRs). The precise mechanisms of the hormone-mediated regulation of stilbene biosynthesis remain unclear, although, in recent years, a number of studies have led to the identification of several transcription factors, including WRKY, NAC, ARF2, and MYB, ERF and a MAPKKK, which could be involved in the complex transcriptional network of *STS* genes that leads to the biosynthesis of stilbenes in response to a wide range of external stimuli and hormone-related signaling pathways.

3.3.4. Transcription factors involved in the regulation of stilbene biosynthesis

Biosynthesis of the major groups of phenolic compounds including lignins, flavonoids and stilbenes is a finely regulated process both in space and time during plant development and in response to external environmental stimuli. Transcriptional regulation of gene expression seems to be the predominant way by which plants control the production of these secondary metabolites and transcription factors (TFs) represent the main actors in this process.

In recent years, numerous studies identified several TFs, which appear to be involved in the transcriptional regulation of *STS* genes. The majority of these studies were conducted in grapevine, thanks to the availability of a well annotated genome and a high number of transcriptomic data.^{32,257} Höll et al.²⁰¹ identified and characterized two R2R3-type V-myb myeloblastosis viral oncogene homolog (MYB) TFs, namely MYB14 and MYB15, that positively regulate the stilbene biosynthetic pathway. These TFs strongly co-express with several *STS* genes and with the accumulation of *trans*-piceid both in leaf tissues under biotic and abiotic stresses and in the skin and seeds of healthy developing berries during fruit maturation. Transient gene reporter assays in cell suspensions of *V. vinifera* and tobacco, showed that TFs specifically activate the promoters of two *STS* genes, namely *VvSTS29* and *VvSTS41* and the ectopic expression of *MYB15* in grapevine hairy roots resulting in increased *STS* expression and piceid accumulation *in planta*. Taken together, these results strongly suggest the involvement of MYB14 and MYB15 TFs in the induction of at least two *STS* genes in *V. vinifera*.

The strong co-expression between *MYB14* and *STS* genes in grapevine leaves exposed to UV-C light was confirmed by Fang et al.²⁵⁸, who showed that the variation in *MYB14* expression correlates with the variation in resveratrol content in two grapevine cultivars characterized by different levels of stilbene accumulation: the high-resveratrol producer 'Z168' (*V. monticola* × *V. riparia*) and the low-resveratrol producer 'Jingzaojing' (*V. vinifera*). Using a one-hybrid yeast assay, authors also demonstrated that MYB14 directly binds the *STS* promoter (Box-L5 motif) *in vitro* and confirmed that a transient overexpression of *MYB14* activates *STS* expression in grapevine leaves. Finally, the same authors showed that *MYB14* overexpression in transgenic Arabidopsis can activate GUS expression driven by an *STS* promoter.²⁵⁸

The relation between the transcriptional regulation exerted by MYB14 and the grapevine signaling pathways related to JA, ROS and calcium was recently investigated by Duan et al.²⁵⁹ and Luo et al.²³⁰ Duan et al.²⁵⁹ showed that a NADPH oxidase RBOH-dependent oxidative burst, a Ca²⁺ influx, a MAPK cascade, and JA signaling but not SA are involved in the transcriptional activation of *MYB14*. Conversely, Luo et al.²³⁰ demonstrated that the promoter activity of *VqMYB14*, the *MYB14* orthologous from Chinese wild grape *V. quinquangularis*-Pingyi (*V. quinquangularis*-PY), is induced as part of both basal immunity (PTI) and effector-triggered immunity (ETI), by the elicitors flg22 and harpin, respectively, and is associated not only to a change in Ca²⁺ cytosolic concentration and to an oxidative burst, but also to treatment with SA. Authors proposed that calcium influx acts as a positive modulator of the SA-dependent signaling pathway activating the *VqMYB14* promoter under infection. Interestingly, SA is able to significantly activate the promoter of *VqMYB14*, but not the promoter of its *V. vinifera* orthologous *VvMYB14*. This evidence suggests that SA could act on stilbene biosynthesis in a genotype-dependent manner.

Together with *MYB14* and *MYB15*, a third paralogous gene, namely *MYB13*, could be involved in the regulation of stilbene biosynthesis. From a systems-oriented analysis based on a gene co-expression network (GCN) build on a multitude of microarray and RNA-seq grapevine expression data, this TF seems to share common feature with MYB14/15.²⁶⁰ Nevertheless, so far, no functional demonstration of its involvement in *STS* gene regulation has been produced. A recent study has identified another MYB TF, VaMyb1, which appears to negatively regulate resveratrol biosynthesis in the cells of *V. amurensis*. Its expression was found to be inversely related to the level of resveratrol produced in cell cultures of *V. amurensis* and its overexpression in resveratrol-producing callus cell cultures of *V. amurensis*, was associated to a decrease in resveratrol content.²⁶¹

The biosynthesis of stilbenes and the regulation of their biosynthetic genes have also been related to the mitogen-activated protein kinase (MAPK) signaling cascade. A Raf-like MAPKKK gene, *VqMAPKKK38*, was isolated and functionally characterized from *V. quinquangularis* accession 'Danfeng-2'.²⁶² This gene is induced in response to a range of stresses, including powdery mildew infection, high salinity, chilling, and treatment with hormones such as SA, MeJA, ET and ABA. Transient overexpression and knockout in grapevine leaves indicated that *VqMAPKKK38* probably controls *STS* transcription and stilbene biosynthesis by regulating the activity of *MYB14*. The observation that *VqMAPKKK38* expression is induced by hydrogen peroxide (H₂O₂) and calcium influx suggests that this TF acts through calcium signaling and ROS signaling pathways.²⁶²

AREB/ABFs are ABA-responsive TFs involved in several processes including plant responses to abiotic stresses, germination and ripening. These regulators contain a basic leucine zipper family (bZIP)-type DNA-binding domain that binds the ABA responsive element and mediates the ABA-dependent gene activation.²⁵⁴ One of these grapevine TFs, *ABF2*, is strongly expressed in the berry, from the onset of ripening to the harvest stage, and is up-regulated by ABA treatment. Overexpression of this TF in grapevine cell cultures boosts the production of resveratrol and piceid, making it a potential candidate for the ABA-mediated regulation of stilbene biosynthesis in grapevine.²⁵⁴

By constructing a large-scale gene coexpression network (CGN) based on most of grapevine microarray and RNA-seq data available from public repositories, Vannozzi et al.²⁰² identified a

number of novel candidate TFs putatively involved in *STS* regulation and belonging to other gene families than MYBs. Among these were four WRKY TFs, namely VviWRKY03, VviWRKY24, VviWRKY43 and VviWRKY53, which represent one of the most enriched TF families in terms of correlation frequencies with *STS* genes. Expression analyses under both unstressed and stressed conditions, together with gene reporter assays, suggested different hierarchies for these TFs in the regulation of the stilbene biosynthetic pathway. Results indicated that VviWRKY24 has a direct effect on the promoter activity of *VviSTS29* and could be involved in the regulation of stilbene biosynthesis in response to biotic stresses, considering that its Arabidopsis orthologue, AtWRKY33, is involved in the regulation of the PTI response in Arabidopsis.²⁶³ Conversely, VviWRKY03 acts through a combinatorial effect with VviMYB14, suggesting that these two regulators may interact at the protein-protein level, probably through the mediation of other 'bridge' proteins such as WD40s and bHLHs, as already observed in Petunia.²⁶⁴

Another TF belonging to the WRKY family, VvWRKY8, was recently found to be a negative regulator of the stilbene biosynthetic pathway. This TF is able to physically interact with VvMYB14 proteins through their *N*-terminal domains to prevent them from binding to the VvSTS promoter. Its transient and stable overexpression in grapevine results in decreased expression of two *STS* genes (*VvSTS15* and *VvSTS21*) and *VvMYB14*, as well as in a reduction of resveratrol accumulation. Moreover, this TF seems to be induced by application of exogenous resveratrol being part of a regulatory loop that may be an important mechanism for the fine-tuning of stilbene biosynthesis in grapevine.²⁶⁵

To conclude, there is accumulating evidence that relates variations in the transcriptional activity of *STS* genes with epigenetic modifications linked to DNA methylation. Treatment of grapevine cell cultures with 5-azacytosine, a DNA demethylating agent, has revealed that the higher the level of cytosine DNA methylation within particular *STS* genes, the lower the level of resveratrol in the cell cultures.^{261,266} More recently, using bisulfite sequencing, Tyunin and Kiselev demonstrated that there is a significant decrease in the methylation level of three *V. amurensis* genes (*VaSTS2*, *VaSTS6* and *VaSTS10*) induced under UV-C exposure, whereas there are no changes in the methylation pattern of the constitutively expressed *VaSTS1* gene.²⁶⁷

4. Antifungal activity of stilbenes in plants

The stilbene bioactivity spectrum is broad as these compounds display biological effects towards all living organisms including viruses, bacteria, fungi, plants and also animals. Phytostilbenes can intervene in allelochemical processes inhibiting seed germination and photosynthesis in plants.^{268,269} Besides, stilbenes take part in the constitutive defense mechanisms of plants displaying deterrent activity to herbivores in green alder (*Alnus crispa*),²⁷⁰ nematocidal properties in pines (*Pinus massoniana*, *P. strobes* and *P. palustris*)²⁷¹ as well as insecticidal capacities, for instance, against the Fall Armyworm (*Spodoptera frugiperda*).²⁷² Stilbenes have also proven to intervene in the constitutive defenses of *Pinus* spp. towards wood decay,²⁷³ but the most recognized role of stilbenes probably is their contribution to the active defense of plants as phytoalexins. We have deliberately chosen to focus on this specific aspect of the biology of stilbenes. *Phytoalexins* are small defensive molecules displaying biocidal activity and whose *de novo* synthesis is stress-dependent.¹⁶ The

production of a phytoalexin corresponds to a transient phenomenon by which a pathogen is confronted during a short time to the action of a toxic compound. Some works have indeed reported that stilbene synthase mRNAs are produced in two successive waves spaced by one to five days following stress exposure and decreasing thereafter.²⁷⁴⁻²⁷⁷ To meet the definition of a phytoalexin, a given compound should: 1) be of low molecular weight (from 200 to 900), 2) be synthesized as a response to biotic and/or abiotic stresses from remote precursors and 3) display antimicrobial activity generally in the range of 10 to 100 μM .

4.1. Antifungal activity of stilbenes

For evaluating the toxicity of any compound, it is important to have appropriate bioassays. The well-known low water-solubility of resveratrol and its derivatives has often led to a misestimating of their antifungal activity and mainly to underestimate their actual toxicity. Thus, use of organic solvents such as ethanol, dimethylsulfoxide or acetone at concentrations of 1 up to 5% is often needed to ensure the solubility of stilbene phytoalexins in liquid media allowing accurate determination of their toxicity. Assessment of phytostilbene activity on silica gel TLC plates or solid medium, that is, where they are not in solution, is rarely employed unless in case of newly characterized compounds and poorly-water soluble stilbene oligomers.²⁷⁸

4.1.1. Does resveratrol display a real toxic activity?

In the initial work of Langcake and Pryce¹⁷ which afforded the first evidence of the function of resveratrol as a phytoalexin from the Vitaceae, this compound was proven to display low fungitoxicity. Concentrations higher than 870 μM leading to 50% of spore germination inhibition (ED_{50}) were recorded for *Cladosporium coccumerinum*, *Botrytis cinerea* and *Pyricularia oryzae* as well as towards the release and the mobility of zoospores from *Plasmopara viticola* (Table S1). The methodology employed for the germination tests did not include the use of any organic solvents for resveratrol solubilization. In contrast, a significant inhibiting effect of resveratrol on the fungal growth of *B. cinerea* (438 μM), *P. oryzae* (276 μM) and *Fusarium oxysporum* (88 μM) was observed on agar containing acetone at a final concentration of 1%.¹⁷ These data have been revisited by using tests in which resveratrol was added to the medium with ethanol at a concentration of 4%. In this case, a complete dissolution of this compound was ensured, as evidenced by observing *B. cinerea* conidial suspensions under inverted light microscopy.^{117,279} In this case, the ED_{50} recorded for resveratrol was 390 μM , that is, more than half concentration of that previously reported,¹⁷ this compound being lethal at 700 μM . Others but contradictory results have been reported more recently regarding the potent effect of resveratrol towards the inhibition of the germination of *B. cinerea* conidia. Caruso et al.²⁸⁰ noted a 70% inhibition at a concentration of 438 μM though Wen et al.²⁸¹ published an ED_{50} of 1153 μM . These discrepancies illustrate the difficulty of evaluating the real fungitoxicity of resveratrol *in vitro* and put in question the role of resveratrol as a phytoalexin.

Resveratrol activity was also determined with many other pathogenic microorganisms.²⁸²⁻²⁸⁷ Studies found ED_{50} and ED_{100} of respectively 192 μM and 500 μM for zoospore mobility towards *P. viticola*^{282,286} though a higher ED_{50} of 484 μM was reported regarding sporulation of the same fungus (Table S1).²⁸⁷ The inhibitory activity of resveratrol was evaluated on the mycelial growth of various

fungi associated with the esca disease of grapevine indicating that, at a concentration of 490 μM , this compound was able to affect the development of certain pathogens such as *Stereum hirsutum* and *Fomitiporia punctata* though being ineffective on *Libertella blepharis* and even acting as a stimulator of the growth of *Phaeomoniella chlamydospora* or *Phaeoacremonium aleophilum*.²⁸³ Resveratrol displays significant activities with regard to the inhibition of the development of yeast and filamentous fungi decreasing by 57.2% the growth of *Saccharomyces cerevisiae* and by respectively, 16.1 and 36.4%, those of *Penicillium expansum* and *Aspergillus niger* at the low concentration of 48 μM .²⁸⁴ Finally, resveratrol was unable to counteract the radial growth of various wood decay fungi at a concentration of 438 μM (Table S1).²⁸⁵

Altogether, the afore-mentioned data reveal that resveratrol shows ED_{50} of hundred micromoles, that is, out of the values commonly accepted for phytoalexin activity which falls within the range of 10 to 100 μM .¹⁵ This compound should thus be considered as a stress metabolite, precursor of more active compounds, rather than as a phytoalexin.¹⁷

4.1.2. Antifungal activity of other stilbene monomers

The biocidal activity of the biosynthetically related resveratrol derivatives was also assessed with pterostilbene being the most studied compound. Pterostilbene has been characterized as a phytoalexin from grapevine by Langcake's group (Figure 2).¹¹² This dimethylated stilbene displays low ED_{50} values regarding the inhibition of the spore germination of *B. cinerea* (70 μM) or *P. oryzae* (35 μM).²⁸⁸ Even lower concentrations, respectively of 17.6 and 9 μM , were obtained for the 50% inhibition of sporangia release and zoospore mobility in *P. viticola* (Table S1).²⁸⁸ Since then, further works have confirmed the high fungitoxic activity of this compound leading to the same ED_{50} values towards the germination of *B. cinerea* conidia (70 μM ²⁸⁹ and 78 μM),¹¹⁷ the zoospore mobility in *P. viticola* (14.6 μM)²⁸² or the mycelial growth of esca associated fungi of grapevine (39 to 390 μM) (Table S1).²⁸³ Pterostilbene may thus be considered as being five to ten times more active than resveratrol depending on the tested pathogen. It ensues that methylation of the hydroxyphenyl groups of stilbenes increases their biological activity. For example, piceatannol (254 μM) and astringin (705 μM) display 2 to 2.5 higher ED_{50} values regarding inhibition of mildew development than their respective methylated derivatives, isorhapontigenin (116 μM) and isorhapontin (272 μM) (Figure 2).²⁹⁰ Increase in the biological activity of methylated stilbenes compared to hydroxystilbenes has been discussed.^{280,286,291} It was suggested that the higher antifungal activity of three 2-furan ring containing-methylated stilbene analogues (3,4,5-trimethoxy- β -(2-furyl)-styrene, 3,5-dimethoxy- β -(2-furyl)-styrene and methoxy- β -(2-furyl)-styrene) (for structures see Figure 2) towards *B. cinerea* vs resveratrol, was linked to the presence of the methylated groups conferring these derivatives a higher hydrophobicity as determined by their log P values (> 4 for the former and 3.0 for resveratrol).²⁸⁰ Log P measures the differential solubility of a given compound between an organic solvent and water. The highest the log P, the highest the hydrophobicity. In fact, the high lipophilic character of a given phytoalexin determines its ability to penetrate cellular membranes.²⁹¹ A lower degree of hydroxylation may likely account for the observed higher toxicity of pinosylvin compared to resveratrol with regard to the mycelial growth of several wood decay fungi.^{273,285} At the ultrastructural level, pterostilbene and resveratrol fungitoxicities towards *B. cinerea* are marked by a

rapid destruction of the plasma membrane and all membranes of the endocellular system, disappearance of ribosomes, mitochondria disorganization^{279,292} as well as a rapid cessation of conidial respiration.^{280,292}

4.1.3. Antifungal activity of stilbene oligomers

Reports on the antifungal activity of stilbene oligomers are less abundant in the literature, likely due to the difficulty to recover them in suitable amounts to conduct biological tests and to their low aqueous solubility. However, when using the same method as that described for monomeric stilbenes, ϵ -viniferin showed a four times lower ED₅₀ regarding inhibition of *B. cinerea* conidia than resveratrol (220 μ M vs 870 μ M) and a two times higher inhibitory activity on *B. cinerea* mycelial growth (220 μ M vs 438 μ M).²⁸⁸ ϵ -Viniferin displays variable but generally high fungitoxic activity with ED₅₀ towards the sporulation of *P. viticola* of respectively 12.7 μ M²⁸² and 70 μ M (Table S1).²⁸⁷ Glucosylated dimers recently identified from Spruce bark extracts and named piceasides I, J and L were attributed ED₅₀ ranging from 96 to 147 μ M regarding mildew development.²⁹⁰ In the same experiments, ampelopsin A, another dimer, displayed a very low antifungal activity (ED₅₀ of 934 μ M). Surprisingly, highly polymerized stilbenes such as the tetramers hopeaphenol (ED₅₀ of 168 μ M), miyabenol C (ED₅₀ of 40 μ M), vitisin A (ED₅₀ of 20 μ M) and vitisin B (ED₅₀ of 12 μ M) turned out much more fungitoxic towards mildew development, than their monomeric precursor resveratrol (ED₅₀ of 484 μ M) (Table S1).²⁸⁷

4.1.4. Can stilbenes play a significant role in plant-pathogen interactions outcome?

The outcome of the interaction between a plant and a pathogen is determined, in part, by the capacity of the plant to rapidly set up a chemical barrier to the invading host which depends on the speed and the level of phytoalexin biosynthesis as well as the nature of the synthesized phytoalexins. To address this question, one can consider which quantities of stilbene phytoalexins can accumulate at the infection sites. Members of the Vitaceae family have been described as those displaying the highest production of stilbene compounds. Great effort has been expended to determine both the amounts and the nature of stilbene phytoalexins produced as a response to stress or fungal infection. Determining the concentrations of phytoalexins to which pathogens can be exposed is hindered by the maceration of plant tissues that occurs during fungal development. During experiments conducted in the vineyard or *in vitro*, several works have reported a high accumulation of stilbenes in the uninfected zones of grapevine leaves or grape clusters directly surrounding *B. cinerea* lesions thus limiting progression of this pathogen.^{220,293,294} In order to assess the amounts of stilbenes able to accumulate in grapevine leaves or grape berries more accurately, a short UV-irradiation at 254 nm was used to induce their production.¹⁷

Already old studies have reported that resveratrol is synthesized very rapidly (within 24 hours after the UV stress application) and at very high levels ranging from 100 to 1,000 μ g/g fresh weight (FW) in grapevine leaves^{17,295-298} and up to 500 μ g/g FW in grape berry skins.^{297,299,300} Stilbene biosynthesis is localized at the lower side of grapevine leaves where the cuticle is thinner and where stomata which represent entry points for some fungi, are found. In fact, observation of the mesophyll of *V. rupestris* leaves with confocal UV microscopy following a short UV-C irradiation has

revealed an intense blue fluorescence characteristic of the presence of stilbenes throughout epidermal cells and the guard cells of stomata, that is, at the potent penetration sites of the zoospores of *P. viticola*.²⁷⁹ The levels and the nature of stilbenes produced in grapevine varieties or species resistant to biotrophic pathogens such as *P. viticola*, have been described in numerous studies showing that very high concentrations of resveratrol and viniferins^{152,282,301,302} as well as pterostilbene³⁰³ can accumulate in infected grapevine leaves. Production of high amounts of resveratrol at the grape skin level can be justified by the fact that this structure appears as the principal and first barrier to infection.^{294,296,297,299} High resveratrol-producing young berries are able to defend themselves to *B. cinerea* attacks while maturing ones which display low capacity for resveratrol synthesis as a result of a decrease in stilbene synthase gene expression at the late stages of the maturation process,³⁰⁴ are more susceptible to this pathogen.^{297,299}

Understanding the role of stilbene phytoalexins in plant pathogen interactions is also hindered by the difficulties in determining the nature of stilbenes and their real amounts in infected plant tissues. In order to cope with these problems, approaches using Matrix Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry Imaging have been used which allow a sort of mapping of the stilbenes produced upon infection of grapevine leaves with *P. viticola* and their respective distribution at the infection sites.³⁰⁵ Can stilbenes be valuable markers of the resistance of plants to disease? Several studies have already reported a possible link between stilbene phytoalexin production and disease tolerance in grapevine,³⁰⁶⁻³⁰⁹ A good correlation was found between the aptitude of 13 different grapevine species or varieties cultivated *in vitro* to synthesize resveratrol and their estimated field disease resistance.³⁰⁹ A similar interrelationship was described between the production of resveratrol and arachidin-3, two stilbene phytoalexins from peanut pods, and the resistance to insects.³¹⁰

4.2. Fungal inactivation of stilbene phytoalexins: Catabolism and ABC transporters

Plant pathogenic fungi have evolved different mechanisms to counteract the action of phytoalexins. Already in 1980, Mansfield³¹¹ in the book he co-edited with Bailey on "Phytoalexins" evoked an original concept in which the interaction of the fungal pathogen, *Botrytis*, and tissues of soy plants (*Vicia faba*) was seen as a balance between phytoalexin biosynthesis by the plant and suppression of phytoalexin action by the pathogen. Counteraction of the pathogen incorporates both fungal metabolism of phytoalexins and their inactivation through the existence of phytoalexin transporters.¹⁵ It becomes evident that the weighting of this balance depends on the pathogen's capacity to metabolize and/or to inactivate the phytoalexin(s) to which it is exposed.³¹¹

4.2.1. Fungal catabolism of stilbenes

Phytoalexin inactivation resulting from fungal catabolism was suggested early.³¹² At the same time arose the idea that the ability of a phytopathogenic microorganism to metabolize a given phytoalexin may relate to its pathogenicity and aggressiveness.^{313,314} Several studies have underlined the role of fungal catabolism of phytoalexins in the outcome of many plant pathogen interactions. The occurrence of adaptive mechanisms elaborated by fungi to escape from phytoalexin action has

also generated a new research field dedicated to the development of the so-called paldoxins, that is, inhibitors of fungal enzymes involved in phytoalexin detoxification.³¹⁵

Interest for stilbene phytoalexin metabolism by fungi arose in the late 80's. A first study was published establishing a relationship between the ability of various *B. cinerea* isolates to degrade resveratrol and their pathogenicity to grapevine *in vitro*-plantlets.³¹⁶ Laccase was suggested as the enzyme being likely involved in resveratrol catabolism. Two years later, the first evidence of the conversion of resveratrol to a resveratrol dehydrodimer formed in 85% yield by *B. cinerea* filtrates was reported (Figure 9).³⁶ This dehydrodimer also called δ -viniferin, was shown to conserve at least one *trans*-stilbene moiety with a dihydrobenzofuranic structure close to that of ϵ -viniferin. Similar experiments led to the identification of a *trans*-pterostilbene dehydrodimer.¹⁵⁰ Incubation of resveratrol with whole cell cultures of *B. cinerea* also yielded various 8-8' dimers outlined above, such as restrytisols A, B and C, pallidol and leachianols F/G (Figure 9).¹⁵¹ Stilbene dimerization was putatively attributed to the laccase until Pezet¹⁴⁹ achieved the purification of a laccase-like stilbene oxidase of 32 kDa of *B. cinerea* capable of transforming resveratrol into ϵ -viniferin with a 97% yield. Three characterized *B. cinerea* laccases, Bclcc1, Bclcc2 and Bclcc3 were also found to be involved in stilbene oligomerization.³¹⁷ Aside from *B. cinerea* laccases, other laccases from *Myceliophthora* and *Trametes* spp, have been used for the enzymatic production of various stilbene dimers including δ -viniferin, the pterostilbene dehydrodimer, pallidol as well as restrytisol and leachianol in quantities in the order of tens of milligrams with accomplished yields ranging from 0.2 to 59%.^{151,318-321}

Insert Figure 9

The fungal degradation of stilbene monomers was observed in other plant-pathogen systems. The conversion of astringin to various astringin dimers has indeed been reported in the case of the Norway spruce (*P. abies*) fungal parasite, *Ceratocystis polonica* (Figure 10) by Hammerbacher's group.³⁴ Additionally, these authors identified other pathways of stilbene catabolism by this fungus showing that astringin is the substrate of a deglycosylation/polymerization process yielding first piceatannol and piceatannol dimers. Several ring-opened lactones of astringin and piceatannol were identified, as well (Figure 10). Aside from their conversion to dimers or lactones, resveratrol and derivatives can undergo cleavage of the stilbene 1,2-diphenylethylene core through the catalytic action of specific carotenoid cleavage oxygenases (CAOs) from *Ustilago maydis*, the causal agent for corn smut, called resveratrol cleavage oxygenase 1 (UmRco1) and whose heterologous expression in *E. coli* yielded to the production of hydroxybenzaldehydes from resveratrol or piceatannol.³²² The formation of 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde as a result of the oxidative cleavage of the extranuclear double bond of resveratrol was also reported with the carotenoid cleavage oxygenase CAO1 of the ascomycete, *Neurospora crassa* (Figure 9).³²³ Three other CAOs functioning as stilbene cleavage oxygenases (SCOs) have recently been functionally characterized from *Aspergillus fumigatus*, *Chaetomium globosum* and *Botryotinia fuckeliana*³²⁴ as well as a resveratrol cleaving dioxygenase NOV1 from *Novosphingobium aromaticivorans*.^{325,326}

Insert Figure 10

Many degradation pathways of stilbene phytoalexins have been described in fungi involving deglucosylation, dimerization and oxidation reactions leading to lactones or aldehydes. It is thus difficult to draw conclusions as to define a general mechanism for stilbene catabolism in fungi. It seems that the degradation pathways vary from one fungal pathogen to another though, sometimes, they can differ for a given pathogen as in the case of *C. polonica*.³⁴ The significance of stilbene metabolism by fungi is also questionable as it is difficult to ascertain whether the degradation of stilbene phytoalexins does correspond to a detoxification process or simply provides fungal pathogens with a carbon source. The transformation of resveratrol by *B. cinerea*, for example, is supposed to yield a dehydrodimer displaying a higher fungitoxic activity as the monomer itself.³⁶ As stilbene dimers show poor aqueous solubility, the conversion of resveratrol and pterostilbene into dimeric structures was envisaged as a mechanism by which the fungus could escape from the action of the phytoalexin through dimer insolubilization.¹⁵⁰ Moreover, unidentified oxidized compounds of resveratrol accumulated in form of brown pigments in the vacuoles of *B. cinerea* conidia, this process being considered as a survival mode of conidia to the action of resveratrol.³²⁷ The differential capacity of various *B. cinerea* isolates to metabolize resveratrol results in variable virulence levels against grapevine. High laccase-producing isolates (Lac+) with a high capacity for stilbene degradation were the most aggressive whereas the Lac- isolates, well correlating with low stilbene degradation, displayed the lowest pathogenicity to grapevine *in vitro* plantlets.³¹⁶ The same trend was recorded regarding the virulence of *C. polonica* in Norway spruce. Highest aggressiveness towards this plant was in good agreement with the ability of its fungal pathogen to degrade astringin.³⁴ Nevertheless, such a correlation was not observed for Δ CAO-1 mutants of *N. crassa* impaired in CAO activity likely due to the fact that, in this case, resveratrol was not an efficient inhibitor of fungal growth.³²³ Finally, the oxidative degradation pathway of stilbenes leading to either ring-opened lactones or hydroxybenzaldehydes, has been envisaged as a possible way for fungi to increase their carbon supply.^{34,323} Previous works have indeed reported that resveratrol could be utilized as a growth substrate by bacteria of the Rhizosphere.³²⁸

4.2.2. Role of ATP-Binding Cassette (ABC) transporters

Fungal plant parasites have developed a lot of mechanisms to inactivate fungicides and phytoalexins or to escape from their action. One of these mechanisms uses various membrane transporters by which fungal cells are able to extrude the toxic compounds they are exposed to. These belong to two major classes of transporters, the major facilitator superfamily transporters on one hand and the ATP-Binding Cassette (ABC) transporters, on the other. A lot of ABC transporters involved in the efflux of synthetic or natural biocide compounds have already been described in fungi.^{37,329,330} Some of the ABC transporter-encoding genes have been considered as fungal virulence factors.^{38,331-335} Additionally, genes encoding ABC transporters from several fungi were cloned and their role in plant pathogen interactions were elucidated through functional analyses.^{37,330,332,334,336-339} Only few studies have reported on the existence of ABC transporters for phytoalexins and their putative role in phytoalexin inactivation and as virulence factors in fungi.

The first report of an ABC transporter modifying the tolerance of a fungal pathogen to resveratrol was provided by the group of De Waard.³³² The *BcatrB* gene encoding an ABC transporter from *B. cinerea* was cloned. The transporter protein contains 1,139 amino acids with a 31 to 67% identity with the amino acid sequence of many other fungal ABC transporters. *BcatrB* gene expression is up-regulated both with the fungicide fenpiclonil and resveratrol, *BcatrB* replacement mutants being more sensitive to these two compounds. $\Delta BcatrB$ mutants are also less virulent to grapevine leaves underlying the role of this transporter in the pathogenicity of *B. cinerea* to that plant.³³² Another ABC transporter-encoding gene, *BcatrA*, cloned from *Botryotinia fuckeliana* (*B. cinerea*) is up-regulated by numerous chemical fungicides as well as the phytoalexins pisatin, eugenol and resveratrol and thus works as a multi-drug transporter. $\Delta BcatrA$ mutants do not display any decreased virulence on *Botrytis* host plants, however.^{334,336}

A homologue of the gene *BcatrB* called *AtrB* encoding for an ABC multidrug transporter has also been identified from *Aspergillus nidulans*. Disruption of *AtrB* leads to increased sensitivity towards a range of fungicides as well as resveratrol.³³⁷ Two other ABC transporters, *MgAtr5* from the wheat pathogen *Mycosphaerella graminicola* and *PMR5* from *Penicillium digitatum* display a high amino acid sequence homology with the *BcatrB* transporter from *B. cinerea* and the *AtrB* transporter from *A. nidulans*.^{338,339} Disrupting the *MgAtr5* gene results in enhanced sensitivity to resveratrol as well as to a plant defense compound from wheat, resorcinol.³³⁸ Replacement mutants of gene *PMR5* also display an increased susceptibility to numerous fungicides, resveratrol and the alkaloid camptothecin underlying the involvement of this transporter in the multidrug resistance of *P. digitatum*.³³⁹

These examples thus illustrate that plant parasites have evolved diverse mechanisms including catabolic pathways of stilbene phytoalexins as well as ABC transporters playing the role of virulence factors, to counteract their phytotoxic activity.

5. Plant engineering with stilbene phytoalexins

5.1 General aspects

Exploring the possibility of strengthening the defense mechanisms of plants through phytoalexin pathway engineering has generated considerable interest and a lot of hope in the last two decades. Persuasive arguments of the role of phytoalexins arose from the first phytoalexin pathway engineering projects in the 90's.^{39,340} The initial report on the transfer of a complete set of stilbene synthase (*STS*) genes from grapevine to tobacco conferring to that plant the capacity to produce the non-native phytoalexin resveratrol and a higher resistance to the fungal pathogen, *Botrytis cinerea*,³⁹ was seen by the scientific community as a strong encouragement to explore the possibility of reinforcing plant defenses through the manipulation of phytoalexin pathways.³⁴¹ As aforementioned, resveratrol is formed through a single enzymatic step from *p*-coumaroyl-CoA and malonyl-CoA, which are universal compounds found in all plants. The transfer of only one gene is then sufficient to produce resveratrol in heterologous plants making stilbenes as the first choice for phytoalexin engineering.^{40,41,91} From that point on, the transfer of *STS* genes from different origins was carried out to various plants. Expression of *STS* genes was reported in alfalfa,³⁴² apple,^{343,344} Arabidopsis,^{87,109,177,345} aspen,²⁸⁵ barley and wheat,³⁴⁶⁻³⁴⁹ chinese digitalis,³⁵⁰ grapevine,^{351,352} hop,³⁵³

kiwifruit,³⁵⁴ lettuce,³⁵⁵ oilseed rape,³⁵⁶ papaya,³⁵⁷ pea,³⁵⁸ rice,³⁵⁹⁻³⁶¹ tobacco,³⁶² tomato,^{85,363-367} soybean,³⁶⁸ strawberry,⁸⁶ and white poplar.³⁶⁹

5.2 Choice of stilbene synthase gene and its driving promoter for plant transformation

5.2.1 Stilbene synthase

To date, a lot of *STS* genes have been used for their heterologous expression in plants. The Vitaceae constitute the family generally displaying the highest stilbene production. Moreover, annotation of the grapevine genome has identified 48 *STS* genes revealing a large expansion of the genes involved in resveratrol biosynthesis.^{184,202} Numerous genetic transformations were thus performed using *STS* genes belonging to this plant family. The most commonly employed genes were those initially used for the first transformation experiments with the grapevine stilbene synthases, *Vst1* and *Vst2*.³⁹ The *Vst1* gene alone or in combination with *Vst2* gene has been expressed in various crops (see Table 1).^{343,344,346,348,349,351,353,356-359,362,363} The *StSy* stilbene synthase gene from *V. vinifera* has mainly been used for the transformation of tomato³⁶⁴⁻³⁶⁶ and white poplar.³⁶⁹ Transgenesis experiments with *STS* genes from other *Vitis* species known for their established resistance to fungal diseases were performed, as well, using *V. riparia* and *V. labrusca* *STS* genes (*VRIP*, *pLAB* and *NS-Vitis* 3 genes) for the transformation of kiwifruit³⁵⁴ and strawberry⁸⁶ and *V. pseudoreticulata* for grapevine transformation.³⁵² A cDNA encoding for a stilbene synthase from *Parthenocissus henryana* was also expressed in lettuce.³⁵⁵

The origin of the *STS* genes does not seem to be determinant for high stilbene production in the transgenes. Three *STS* DNA clones, *pVIN* from *V. vinifera*, a low stilbene producer, *pLAB* from *V. labrusca*, a medium stilbene producer and *pRIP* from *V. riparia*, a high stilbene producer, were introduced respectively in kiwifruit,³⁵⁴ but no differences were observed in piceid production within the transgenic plants suggesting that the capacity for stilbene synthesis seems not correlating to the origin of the *STS* gene. This is so far not surprising as limited changes of 11 to 17 amino-acids are found among *Vitis* *STS* genes, none of them affecting the catalytic site which appears to be conserved.⁸⁶ Another interesting resource for *STS* genes is also represented by peanut (*A. hypogea*) whose genes have been used in numerous transgenesis experiments. A lot of *STS* genes from peanut were introduced to various plants: genes *AhRS*, *RS*, *AhRS3*, *AhSTS1*, *PNRS1* and *AhSTS3* (Table 1).^{109,342,350,360,361,367,368} Fewer genetic experiments have been conducted with other *STS* genes, such as the pine pinosylvin synthase gene for aspen²⁸⁵ or the *STS* gene from *Polygonum cuspidatum* for Arabidopsis transformation.⁸⁷ Finally, the *SbSTS1* gene, which was described as the first characterized *STS* gene in monocots,¹⁷⁷ has been expressed in Arabidopsis.³⁴⁵

5.2.2 Promoter choice

Expression of a target gene is largely dependent on the choice of the promoter used to drive its expression. In most of the transgenic experiments reported to date, two major promoters have been used to target *STS* gene expression: the well-known strong cauliflower mosaic virus (CaMV35S) constitutive promoter^{85,87,109,285,342,345,350,352-355,364-367,369} and the UV-wounding-pathogen inducible own promoter of the *Vst1* gene.^{340,343,344,358,359,363} A combination of both the *Vst1* and the *Vst2* promoters or the combination of the inducible p*Vst1* promoter and 4-fold enhancer elements of the constitutive

pCaMV35S promoter was also envisaged as a valuable option to enhance transgene expression in certain studies.^{346,348,349} It has indeed been reported that *STS* gene expression can be strengthened when it is under the control of its own promoter and enhancer elements of the CaMV35S promoter.³⁴⁶ The ubiquitous function of the inducible *Vst1* promoter is illustrated by the fact it is also active in monocots such as rice.³⁵⁹ In grapevine, stilbene biosynthesis is induced by a variety of abiotic stresses, UV-irradiation,^{17,297,370} heavy metals such as aluminum and copper or aluminum-containing fungicides^{209,371,372} and ozone among other factors.^{210,276} Interestingly, the *Vst1* promoter was reported to display an ozone-responsive region differing from the pathogen-inducible sequence.²¹⁰ This promoter is induced by various biotic or abiotic stresses in plants. It has the advantage to trigger a strong stilbene accumulation without any depletion of other interfering metabolic pathways in contrast to plants transformed with transgenes under the control of the pCaMV35S promoter.³⁶²

Other promoters have also been used to target tissues or organs. For example, control of the grapevine *STS Vst1* gene in tobacco by the tapetum-specific promoter *tap1* from *Antirrhinum majus*, yields 100% of male-sterility among the transformed plants, showing that deleterious by side effects of *STS* expression can occur during the use of a tissue specific promoter.³⁶² In contrast, no male-sterility was recorded during expression of the *STS* gene in oilseed rape under the control of the seed specific napin *pnap* promoter.³⁵⁶ Driving the transgene with a fruit-specific tomato lipoxygenase *TomLoxB* promoter in tomato was reported to be responsible of specific *StSy* gene expression in the fruit flesh compared to the CaMV35S promoter.³⁶⁶ Finally, choosing a pathogen-inducible promoter like the alfalfa Pathogenesis-Related protein 10 *PR 10* promoter appears as a better option as it can lead to a 5-100 fold increased resveratrol accumulation in transgenic grapevine without any detrimental effects on male-fertility or plant morphological features.³⁵¹

5.3 Stacked gene introduction

Stacking several transgenes could be an interesting option for plant disease resistance engineering. This can be achieved by direct transformation with a construct containing two or more transgenes or by using a breeding strategy in which hybrid plants are obtained by crossing parental lines constitutively expressing one transgene, respectively. Such an approach has already been developed in transgenic peas obtained from conventional crossing of two transgenic lines, one bearing a polygalacturonase inhibiting protein *PGIP*-encoding gene from raspberry and the other one carrying the stilbene synthase *Vst1* gene from grapevine.³⁵⁸

Co-expression of genes encoding for enzymes involved in stilbene biosynthesis has been reported for the *in planta* production of the potent antifungal phytoalexin pterostilbene.^{109,368} In a first approach, a binary vector was designed for the simultaneous expression of the stilbene synthase *AhSTS3* gene from peanut and the *O*-methyltransferase *SbOMT3* gene from sorghum,¹⁰⁹ leading almost exclusively to the formation of pterostilbene in tobacco and Arabidopsis plants. Accumulation of this highly fungitoxic compound in transgenic tobacco and Arabidopsis ranged from 8 to 52 µg /g FW (Table 1), which is higher than the maximum pterostilbene levels previously recorded in UV-irradiated grapevine leaves (0.2 to 14 µg/g FW).³⁷⁰

In another approach, the combined expression of the *STS AhRS3* from peanut and the resveratrol-*O*-methyltransferase *ROMT* from grapevine¹⁰⁶ was carried out in soybean hairy roots to target the production of pterostilbene.³⁶⁸ Using an *AhRS3-ROMT* gene construct, resveratrol accumulated in hairy roots at concentrations from 30 to 110 µg/g FW and pterostilbene only at 5-8 µg/g FW. Stilbene biosynthesis in the transgenic lines was associated to an increased resistance of soybean roots to root necrosis caused by *Rhizoctonia solani* (Table 1). Interestingly, the production of both resveratrol and pterostilbene resulting from the expression of both genes led to a lower root necrosis level (0-7%) than transgenic lines only expressing the *AhRS3* gene (20-50%). The co-expression of two or more transgenes reveals to be very useful when the transfer of a unique *STS* gene is found to be insufficient to confer plant resistance to a given pathogen.

Stacked gene insertion could also be used to control upstream pathways and increase the availability of metabolic precursors for stilbene biosynthesis. In oilseed rape, for example, the pathway leading to sinapate ester metabolism may divert the utilization of *p*-coumarate through the synthesis of sinapate prior to sinapoylglucose and the ending sinapate esters.³⁵⁶ To limit a possible competition for *p*-coumarate utilization in the seeds for the formation of stilbenes or sinapate esters, one plasmid was designed to silence the *B. napus* glucosyltransferase 1 *BnGST1* gene and the other one for the seed-specific overexpression of the stilbene synthase *Vst1* gene.³⁵⁶ Reducing the transformation rate of sinapate to its glucosyl-derivative through *BnGST1* gene silencing was proven to be an efficient way to channel the metabolic flux (*p*-coumarate) to the stilbenoid pathway. A strong accumulation of the phytoalexin piceid in the order of hundred micrograms per gram of fresh weight was thus observed concomitantly with a decrease in the sinapate ester content in the transformed lines.³⁵⁶

5.4 Effects of *STS* gene expression in engineered plants in terms of phytoalexin production and disease protection

Stilbene synthase gene expression leads to an ectopic accumulation of resveratrol and piceid in numerous plants ranging from one to several hundreds µg/g fresh or dry weight (FW, DW) in leaves, fruits, stems, seeds or roots (Table 1).^{39,85,87,349-351,353,354,356,362,363,366-368,369} All these wild-type plants except the grapevine, do not synthesize stilbenes underlining the fact that the transfer of the single *STS* gene is sufficient to ensure production of these compounds in recombinant plants. The aptitude for stilbene biosynthesis is also related to the species of the targeted plant and ultimately to the endogenous enzymatic pool as well as the bioavailability of the precursors. Consecutive accumulation of piceid or uncharacterized resveratrol glucosides following *STS* gene expression as observed in some plants, is likely the consequence of a further glucosylation process of resveratrol by endogenous glucosyltransferases (see section 2).^{85,87,342,344,349,350,353,354-357,360,364,366,367,369} The methylated resveratrol derivative, pterostilbene, can also be recovered in moderate amounts when specific resveratrol-*O*-methyltransferase genes, *SbOMT3* or *ROMT*, are added to *STS* genes (Table 1).^{109,368}

It is generally difficult to reach conclusions with regard of possible relationships between the expression level of *STS* genes and stilbene accumulation in the transgenic lines. There are some examples of a very low production of resveratrol or piceid in transformed plants (5 µg/g FW in pea

leaves,³⁵⁸ and 3 µg/g DW in rice seeds³⁶¹) or even an undetectable production of these stilbenes in rice and aspen.^{285,359} In fact, *STS* transcription activity can be somewhat altered by modifications occurring during introduction of the transgene and possibly resulting in multiple copy insertion.⁴⁰ Some works clearly established a relationship between the number of transgene copies and levels of stilbene accumulation^{87,343,356,358} though others did not.^{354,357} High piceid-producing lines in transgenic oilseed rape correspond to those having integrated a single copy of the transgene.³⁵⁶ Similarly, lines harboring one copy of the *Vst1* gene in transgenic peas produced more resveratrol than those bearing two copies.³⁵⁸ Other studies are in line with these findings reporting a positive correlation between the low levels of the *STS* transcripts or *STS* proteins and a high copy number of the transgenes.^{87,343,369}

Stilbene accumulation has been described in all organs of plants (leaves, stems, roots, flowers, fruits and seeds) (Table 1). A relationship between the type of organ, the age of the organs and the maturation stages of fruits, on one hand, and stilbene production levels on the other, has also been reported. A kind of variable spatiotemporal distribution of stilbenes is thus observed. In transgenic tomato, piceid accumulates in higher amounts than the aglycone in the fruit peel (126 µg/g FW vs 48 µg/g FW).⁸⁵ According to the promoter used, piceid production can significantly differ in tomato fruits. The TomLoxB promoter induces *STS* gene expression in the fruit flesh whereas the CaMV35S promoter is responsible for major *STS* expression in the skin.³⁶⁶ Still in tomato, higher piceid amounts were recorded in the skin.^{85,366} Leaves from transgenic hop were reported to contain 10-fold lower stilbene amounts than mature bracts.³⁵³ *STS* gene expression may also be dependent on the age of the organ or the ripening stage in fruits. In tomato, stilbene accumulation increases during fruit ripening, with the highest levels of resveratrol and its glucoside being found in ripe red fruits.^{85,364,366} Conversely, young leaves of transformed tobacco, alfalfa and kiwifruit were shown to produce higher amounts of resveratrol or piceid, respectively.^{39,342,354}

5.5 Effects of stilbene pathway expression in transgenic plants: impact on plant development and metabolism and biological inputs

5.5.1 Impact on plant development and plant metabolism

Only few studies mentioned a detrimental impact of *STS* gene transfer on plant development. A marked effect of stilbene synthase gene expression on plant development has early been reported by Fischer et al.³⁶² who observed a 100% male-sterility in transgenic tobacco during transformation with the *Vst1* gene under the control of a CaMV35S two-fold enhancer or the tap1 tissue-specific promoter of *A. majus* (Table 1). An only very partial restoration (10%) of flower fertility was recorded after spraying the plants with flavonoid compounds (kaempferol or isorhamnetin). This observation together with others evidences the proclaimed role of flavonoids in the development of functional pollen.³⁷³⁻³⁷⁵ We have seen in subsection 2.1 that *STS* gene overexpression can compete with the flavonoid biosynthetic pathway regarding precursor availability as stilbenes are compounds sharing with flavonoids the same initial pathway, thus leading to a decrease in the flavonoid, flavonol and anthocyanin contents in various transgenes (Table 1).^{85,86} However, beside a plausible channeling of the precursors towards the stilbene route, the observed decrease in the flavonol content in recombinant strawberry has also been related to a possible *CHS* gene silencing through RNA

interference consequently to *STS* gene overexpression in transgenic strawberry.⁸⁶ This could also account for the surprisingly increased susceptibility to *B. cinerea* observed in that plant relative to the depletion of the production of flavonols, compounds which play an important role in the resistance of strawberry to disease.⁸⁶

5.5.2 Benefits of *STS* engineering in plants

Molecular engineering of the stilbene biosynthetic pathway has been seen as a valuable strategy to improve plant disease resistance as well as to enhance the nutritional value of fruits, cereals and vegetables.⁴¹ The phytoalexin potential to improve plant resistance was first assessed in transgenic tobacco engineered with two grapevine *STS* genes whose expression resulted in high production of the non-native resveratrol phytoalexin.³⁹ However, as the authors themselves admitted, this ectopic synthesis of resveratrol did not confer a total immunity to the fungal pathogen, *B. cinerea*, as only two thirds of the obtained transgenic lines displayed enhanced disease resistance (Table 1).

Most of the data published so far have confirmed that the disease resistance of plants transformed with *STS* genes was not total and that disease symptoms were only reduced in rice towards *P. oryzae*,³⁵⁹ in grapevine towards *B. cinerea*,³⁵¹ in aspen towards wood decay *Phenillus tremulae*,²⁸⁵ in papaya towards bud rot *P. palmivora*³⁵⁷ and in Arabidopsis towards *Colletotrichum higginsianum* (Table 1).⁸⁷ Very variable levels of resistance varying from immunity to high susceptibility to downy mildew, were also noted in transgenic wheat.³⁴⁸ In certain cases, variations in disease resistance within the transgenes also depend on the tested pathogen. For instance, stilbene accumulation in wheat yielded a 19-27% reduction of the disease symptoms caused by *Puccinia recondita* but a 42-71% reduction of the disease symptoms caused by *Septoria nodorum*.³⁴⁹ In the same way, resveratrol production in tomato resulted in a 38-68% reduction of the disease symptoms induced by *P. infestans* though no enhancement in the tolerance to *B. cinerea* and *Alternaria solani* was observed.³⁶³ Unexpectedly, no increased resistance to *B. cinerea* was reported in transgenic kiwifruit³⁵⁴ nor in transgenic white poplar towards rust disease (*Melampsora pulcherrima*)³⁶⁹ despite a high stilbene accumulation (Table 1). This puts in question the antifungal role of monomeric hydroxylated stilbenes like resveratrol. At the opposite, several cases showing a high resistance to disease were described. For example, the mortality rate caused by the root disease (*Fusarium oxysporum*) was only of 2% in resveratrol glucoside-accumulating transgenic lines compared to 57% in the non-transformed ones.³⁵⁰ Hipskind and Paiva³⁴² also reported the absence of pycnidia formation and resistance to *Phoma medicaginis* in alfalfa leaves. Finally, a two-gene transfer including an *STS* gene and a *ROMT* gene in soybean leading to the production of the very potent phytoalexin, pterostilbene, was shown to decrease root necrosis caused by *R. solani*.³⁶⁸

Engineering the stilbene pathway in plants has also found application in the field of functional food through the production of plants with increased nutritional value. Most of the studies published to date have concerned the enhancement of the antiradical and antioxidant activities of transgenic tomato.³⁶⁴⁻³⁶⁶ As an unexpected and indirect effect of the genetic transformation, an increase in the ascorbic acid and glutathione contents in the fruits was also noted contributing to their antioxidant capabilities.^{364,366} Some important features displayed by transgenic tomato fruits

were reported such as a decrease of the phorbol ester-induced pro-inflammatory effects in monocyte macrophages³⁶⁶ or improved cardiac performances (reduction of the size of the myocardial infarct) during feeding rats with transformed tomato.³⁶⁵ Other works have also described an improvement in the lipid and cholesterol blood level profiles in mice fed with transgenic rice.³⁶⁰

Finally, knowledge on the control mechanisms involved in stilbene biosynthetic pathways has made great progress as many transcription factors regulating stilbene synthase have been identified so far (section 3).³⁷⁶ The recent discovery of a regulatory loop negatively controlling stilbene synthase in grapevine,²⁶⁵ opens new ways for metabolic engineering as the existence of such a regulatory system could explain why overexpression of stilbene synthase in transgenic plants does not always result in high resveratrol production.

6. Metabolic engineering of stilbene biosynthesis in microorganisms

6.1. Metabolic engineering of microbes for stilbene production

For a long time, microorganisms have extensively been used for various value-added chemicals and compounds with numerous applications in the food, chemical and pharmaceutical industries.^{44,377-381} Microbial production has some advantages such as low-cost value, rapid growth, easy to grow in scaled-up fermenters and genetic tractability over the plant synthesis.^{43,382} With the development of metabolic engineering tools including pathway engineering, protein engineering, alternative enzyme selection, central carbon flux redirection and synthetic biology approaches, a number of natural and unnatural metabolites have been produced in microbial cell factories.³⁸³⁻³⁸⁵ As stilbenes display various biological activities with potent applications, these compounds and especially resveratrol have been the subject of much interest in the past years. Various studies have been focusing on engineering prokaryotes and eukaryotes to produce stilbenes, such as yeast *Saccharomyces cerevisiae*, yeast *Yarrowia lipolytica*, *Escherichia coli*, *Corynebacterium glutamicum*, and other microorganisms.^{44,386-388} In the following paragraphs, we will summarize such metabolic engineering efforts for the recombinant production of stilbenes.

6.2. Metabolic engineering of stilbenes in yeast

Yeast has been an ideal host for metabolic engineering to produce plant secondary metabolites in recent years.³⁸⁹⁻³⁹² The yeast expression system offers a number of distinct advantages such as higher resistance to low pH, high osmotic stress, the ability to posttranslationally modify eukaryotic proteins and to functionally express membrane-bound cytochrome P450 enzymes. Moreover, yeast is generally viewed as food-safe microorganism, allowing its use in food fermentation to improve nutritional value.^{43,44,92,393} Hence, the yeast platform has been widely developed for stilbene production through metabolic engineering strategies.

Resveratrol is an iconic representative example for engineering of the stilbene pathway in yeast (Figure 11). In order to enhance resveratrol production during fermentation in both white and red wines, Becker et al.³⁹⁴ engineered the wine yeast *S. cerevisiae* FY23 to co-express the coenzyme-A ligase (*4CL216*) from hybrid poplar and *STS* from grapevine, resulting in piceid production from *p*-coumarate. Although the yield of piceid was relatively low in comparison to those found in wines, it provided a successful example of heterologous biosynthesis of resveratrol through reconstructing a

stilbene biochemical pathway in yeast (Table S2).³⁹⁴ Since then, introducing the selective genes of *4CL* and *STS* from different plant sources has been the strategy employed for resveratrol production from *p*-coumarate in different heterologous hosts. For example, Beekwilder et al.³⁹⁵ co-expressed the *4CL2* gene from *N. tabacum* and the *STS* gene from *V. vinifera* in *S. cerevisiae* CEN.PK 113-3B, resulting in 5.8 mg/L unglucosylated resveratrol when the culture was supplemented with *p*-coumarate. The level of resveratrol production was much higher than the level of piceid previously reported in yeast.³⁹⁵ Similarly, Shin et al.³⁹⁶ over-expressed *4CL1* from *Arabidopsis thaliana* and *STS* from *A. hypogea* using constitutive glucolytic promoters in *S. cerevisiae* W303-1A, leading to 3.1 mg/L resveratrol production from *p*-coumarate with 14.4 mol% yield. To increase the resveratrol productivity, Sydor et al.³⁹⁷ analyzed the resveratrol biosynthesis from *p*-coumarate in different *S. cerevisiae* strains harboring *4CL1* from *A. thaliana* and *STS* from *V. vinifera* and obtained an industrial Brazilian sugar cane-fermenting yeast capable of producing resveratrol up to 391 mg/L with use of rich medium (Table S2).

Insert Figure 11

Linking genes together to generate a functional fusion protein offers a strategy to increase production yield. The use of fusion proteins can simplify the number of constructs in the heterologous expression system and co-localize the enzymes' active sites closely in a metabolic pathway. Zhang et al.³⁹⁸ engineered the resveratrol biosynthesis pathway using a translational fusion of *4CL1* from *A. thaliana* and *STS* from *V. vinifera* in *S. cerevisiae* WAT11, which produced 5.25 mg/L unglucosylated resveratrol. The metabolic efficiency apparently increased up to 15-fold compared to co-expression of *4CL* and *STS*.³⁹⁸ The mechanism remains unclear, but it is possible that metabolic efficiency increases by channeling intermediates between enzymes. Alternatively, the active sites in close proximity reduce the diffusion distance of intermediates and then increase the catalytic efficiency.³⁹⁹ Synthetic protein scaffolds have been effectively applied for high-level production of glucaric acid and mevalonate.^{400,401} Using a similar strategy, introduction of resveratrol biosynthesis pathway in *S. cerevisiae* WAT11 resulted in a 5-fold increase of resveratrol over the non-scaffolded reference strain, and a 2.7-fold improvement over the production with fusion proteins (Table S2). The mechanism might depend on the interaction between scaffold protein domains and enzymes of the resveratrol biosynthetic pathway.⁴⁰² Though the resveratrol production was relatively low, the results demonstrated synthetic scaffolds could be used for pathway optimization.

In the stilbene biosynthetic pathway, PAL is responsible for the conversion of phenylalanine to cinnamate, and then C4H catalyzes the hydroxylation of cinnamate to *p*-coumarate (Figure 11). C4H is a cytochrome P450 enzyme, which requires a cytochrome P450 reductase (CPR) for electron transfer. Trantas et al. co-expressed heterologous *CPR* from *Populus hybrid* in *S. cerevisiae*, leading to a 4-fold improvement of *p*-coumarate production from phenylalanine,⁴⁰³ which indicated the activity of yeast endogenous CPR was not enough to support maximal substrate fluxes from phenylalanine to *p*-coumarate. Recently, Li et al.³⁸⁷ optimized the electron transfer to the cytochrome P450 monooxygenase through over-expression of *CPRs* from *A. thaliana* and *S. cerevisiae* simultaneously,

achieving a significant improvement of resveratrol production, as well as the methylated resveratrol derivatives pinostilbene and pterostilbene, from phenylalanine or glucose.

Alternatively, the tyrosine ammonia lyase (TAL) can catalyze the conversion of tyrosine directly to *p*-coumarate, bypassing the rate-limiting hydroxylation step and simplifying the biosynthetic pathway (Figure 11).^{404,405} While the *TAL* gene from *Rhodobacter sphaeroides* can form *p*-coumarate from tyrosine *in vitro*, it failed to produce this compound when overexpressed in yeast *S. cerevisiae* despite the high *TAL* transcripts levels. Based on the hypothesis that differences in codon usage between *Rhodobacter* and yeast might limit protein expression, Wang et al.⁴⁰⁶ expressed the codon optimized *TAL* and fused *4CL* from *A. thaliana* and *STS* from *V. vinifera*, leading to an improvement of *p*-coumarate and resveratrol production in *S. cerevisiae*. In a separate study, 4.3 mg/L resveratrol was obtained in *S. cerevisiae* that expressed a codon optimized *TAL* from *Rhodospiridium toruloides*, *4CL* from *A. thaliana*, *STS* from *A. hypogaea* and *ACC1* (acetyl-CoA carboxylase to increase the malonyl-CoA pool) from tyrosine.⁴⁰⁷ A three-enzyme heterologous pathway (consisting of *TAL* from *Rhodotorula glutinis*, *4CL* from *Streptomyces coelicolor*, and *STS* from *V. vinifera*) was assembled into another yeast *Yarrowia lipolytica*, leading to 1.46 mg/L resveratrol production from tyrosine (Table S2).⁴⁰⁸

Malonyl-CoA is a prime precursor of stilbenes and fatty acid biosynthesis. Naturally, most of the malonyl-CoA is directed towards the fatty acid biosynthesis, and thus only a small amount of malonyl-CoA is available for resveratrol biosynthesis in the host strains expressing the heterologous biosynthetic genes.⁴⁰⁹ Acetyl-CoA carboxylase (ACC) is a key enzyme to enhance the carbon flux through acetyl-CoA to malonyl-CoA. The overexpression of ACC resulted in a slight increase in resveratrol titer produced by a recombinant *S. cerevisiae*,⁴⁰⁷ which indicated the small amount of cellular malonyl-CoA and competition use of fatty acid biosynthesis might limit resveratrol production. Hence, introduction of malonate assimilation pathway and the inhibition of fatty-acid synthesis using the antibiotic cerulenin may provide an alternative approach to further improve resveratrol production in yeast.³⁹⁹ It has also been demonstrated that malonyl-CoA production can be improved via abolishing Snf1-dependent phosphorylation of ACC. The over-expression of the phosphorylation-insensitive version of ACC protein (ACC^{S659A, S1157A}) led to a 31% improvement of resveratrol production in yeast.^{386,410,411}

To produce stilbenes directly from simple carbon resources, such as glucose or other carbohydrates, researchers have concentrated on optimizing the synthesis of the aromatic acid precursors, phenylalanine and tyrosine, in yeast. Removing the enzyme feedback inhibition on the shikimate pathway is the most commonly used strategy to enhance the carbon flux to aromatic acids biosynthesis. For this purpose, Li et al.³⁸⁶ overexpressed the insensitive allele of DAHP synthase (*ARO4*^{K229L}) and chorismate mutase (*ARO7*^{G141S}) in resveratrol-producing yeast, leading to a 78% improvement of resveratrol titer compared with the strain incorporating only the resveratrol biosynthesis pathway in batch cultures (Figure 11). Furthermore, integration of multiple copies of the resveratrol pathway onto Ty4 elements of a tyrosine overproducing strain led to 36-fold improvement in resveratrol titer, resulting in a final resveratrol titer of 531 mg/L in fed-batch fermentation.³⁸⁶ Subsequently, Li and colleagues³⁸⁷ applied a pull-push-block strain engineering strategy for a higher level resveratrol titer from glucose; as a result, up to 800 mg/L of resveratrol

was produced in fed-batch fermentation *via* overexpression of cytochrome P450 monooxygenase, precursor optimization, blocking intermediates degradation and pathway integration. In this work, integration of the heterologous methyltransferases from *Sorghum bicolor* and *V. vinifera* into the resveratrol platform strain resulted in the production of methylated resveratrol derivatives, pinostilbene (3,4'-dihydroxy-5-methoxystilbene) and pterostilbene, at final titers of 5.5 mg/L and 34.5 mg/L, respectively.³⁸⁷ This was the first demonstration of *de novo* biosynthesis of pinostilbene and pterostilbene from glucose in a yeast strain, which demonstrated the potential utility of the engineered yeast for diverse stilbene production.

6.3 Metabolic engineering of stilbenes in *E. coli*

From a long time ago, *E. coli* has becoming one of the most widely used microorganism for plant-derived secondary metabolites, such as flavonoids, stilbenoids, alkaloids and terpenoids.⁴¹² Due to the advantages like fast growth, easy to genetic manipulation, compatibility of various synthetic biology tools, a number of studies are dedicated to the heterologous biosynthesis of stilbenes in *E. coli* recently.⁴¹³

Pathway engineering has been one pioneer tool for metabolic engineering *E. coli* to produce value-added bioactive stilbene polyketides. In 2006, Watts et al.⁸¹ introduced a stilbene biosynthesis pathway containing *4CL* from *A. thaliana* and *STS* from *A. hypogea* into *E. coli*, resulting in resveratrol production (>100 mg/L) and piceatannol production (> 10 mg/L) converted from *p*-coumaric acid and caffeic acid, respectively. This was the first time to demonstrate the feasibility of *E. coli* for stilbene biosynthesis directly.⁸¹ Likewise, co-expression of *4CL* from tobacco and *STS* from grapes in *E. coli* BL21 led to 16 mg/L resveratrol production with 5 mM *p*-coumaric acid as precursor.³⁹⁵ Introduction of the *STS* from *A. hypogea* and *Le4CL* from *L. erythrorhizon* with a broad substrate spectrum in *E. coli* resulted in the production of 15 stilbenes derived from different exogenous carboxylic acids.⁸⁹ Various *STS*s from different plant species were characterized by Lim et al.⁴¹⁴ *via* structure-activity relationships, expression efficiency and the ability to synthesize resveratrol. The *E. coli* BW27784 strain harboring *VvSTS* and *At4CL* from *A. thaliana* finally produced 1.3 g/L resveratrol, and the titer reached up to 2.3 g/L after the addition of cerulenin. With the gene fusion technology, co-expression of fusion protein 4CL::RS from *A. thaliana* and *A. hypogea* in recombinant strains resulted in 80.5 mg/L resveratrol titer from 1 mM *p*-coumaric acid with a 35.28% conversion yield. This work provided an alternative strategy on the optimization of resveratrol fermentation to increase the productivity of resveratrol in future studies.⁴¹⁵

Previous works have already demonstrated the feasibility of *E. coli* for resveratrol production *in vivo* using *p*-coumarate as primary precursor. However, *p*-coumarate is expensive and only produced by a few industrial processes in the market. Using *TAL*, *4CL* from *Saccharothrix espanaensis*, *STS* from *A. hypogea*, 1.4 mg/L resveratrol was obtained from tyrosine.⁴¹⁶ Similarly, co-expression of the *RgTAL* from the red yeast *R. glutinis*, *Pc4CL* from *Petroselinum crispum* and *VvSTS* from *V. vinifera* can also lead to resveratrol production from tyrosine.⁴¹⁷ The low conversion efficiency from tyrosine directly to *p*-coumarate is one of the most important limiting factor for high-level resveratrol production in *E. coli*.⁵¹⁴ Wu and colleagues⁴¹⁷ applied the multivariate modular metabolic engineering approach to engineer a non-native synthetic pathway for resveratrol production from tyrosine. The overall

1 pathway was divided into three modules, including the module I with *TAL* and *4CL* for *p*-coumaroyl-
2 CoA, the module II with *STS* for resveratrol, and the module III with *matB*, *matC* for malonyl-CoA
3 production. With the best strain, the authors were able to achieve 35.02 mg/L resveratrol from
4 tyrosine.⁴¹⁷ TcTAL from *Trichosporon cutaneum* displayed a higher activity towards tyrosine in
5 comparison with RgTAL from *R. glutinis*.⁴¹⁸ Wu et al.⁴¹⁹ applied the strategy that modifies 5' region of
6 mRNA secondary structure to improve TcTAL expression, resulting in 304.5 mg/L resveratrol finally in
7 *E. coli*.

8 To enhance the pool of malonyl-CoA, two strategies such as repression of fatty acid biosynthesis
9 to reduce the consumption of malonyl-CoA and increasing the carboxylation of acetyl-CoA to
10 improve intracellular malonyl-CoA have been most commonly used for stilbene biosynthesis in *E.*
11 *coli*. Lim et al.⁴¹⁴ added the cerulenin, an inhibitor of the *fabB-fabF* gene products from the fatty acid
12 biosynthesis pathway to reduce the consumption of malonyl-CoA, leading to 2-fold increase of
13 resveratrol to 65 mg/L. However, the cerulenin is too expensive to apply for industrial-scale
14 resveratrol production. Instead of cerulenin, overexpression of *ACC* and a biotin ligase from
15 *Photorhabdus luminescens* resulted in an improvement of resveratrol titer, up to 46 mg/L. Antisense
16 RNAs (asRNAs) are single-stranded RNAs that can complementarily pair with their target mRNA and
17 inhibit gene expression, which is a powerful tool for metabolic engineering use.⁴²⁰ Yang et al.⁴²¹
18 employed synthetic asRNA to down-regulate *fabD* gene expression involved in fatty acid
19 biosynthesis, leading to a 4.5 fold increase in intracellular malonyl-CoA concentration, and obtained
20 an improvement of resveratrol production by 1.7 fold in *E. coli*.

21 The clustered regularly interspaced short palindromic repeats interference (CRISPRi) is an
22 emerging gene repression system similar with antisense RNAs system. Application of CRISPRi for the
23 repression of *fabD* gene encoding malonyl-CoA-ACP transacylase resulted in 47.5 mg/L pinosylvin
24 with a 1.9-fold enhancement in yield in engineered *E. coli*.⁴²² After that, Wu et al.⁴²³ constructed a
25 CRISPRi system to enhance malonyl-CoA concentration *via* repressing gene expression with anti-
26 *fabF/fabB/eno/adhE/fumC/SucC* sgRNA, obtaining the highest titer of 281 mg/L pinosylvin in the
27 recombinant strain. It was also reported that combinatorial genetic perturbations by repressing *fabF*,
28 *fabI*, *fabB* and *fabD* with CRISPRi system led to the highest resveratrol titer (187.1 mg/L). The gene
29 *fabI*, encoding the enoyl-acyl carrier protein reductase, plays a vital role in fatty acid biosynthesis.
30 Salas-Navarrete et al.⁴²⁴ explored a strategy based on the reduction of *fabI* expression *via* deleting
31 the -35 promoter sequence of gene *fabI*, resulting in 52.67 mg/L pinosylvin with a 1.5-fold
32 improvement. Previously, an *E. coli* strain with higher carbon flux to acetyl-CoA and malonyl-CoA was
33 developed *via* carrying out genetic interventions predicted by OptForce framework.⁴²⁵ Accordingly,
34 Koffas' group applied the OptForce model for malonyl-CoA accumulation by overexpression of the
35 pyruvate dehydrogenase multienzyme complex, the phosphoglycerate kinase, the glyceraldehyde-3-
36 phosphate dehydrogenase and deletion of fumarase in order to increase the availability of malonyl-
37 CoA instead of using cerulenin, giving 1.6 g/L resveratrol with a 60% increase in yield.^{426,427} In
38 addition, overexpression of malonate assimilation pathway genes *matB* and *matC* from *Streptomyces*
39 *coelicolor* A3(2) and acetyl-CoA carboxylase complex genes (*accCADB*) from *E. coli* BL21(DE3) and
40 *Nocardia farcinica* IFM10152 can also enhance the pool of intracellular malonyl-CoA and acetyl-CoA
41 towards resveratrol and piceatannol in engineered *E. coli*.¹²⁶

1 In previous studies, engineered *E. coli* typically produced stilbenes from tyrosine, phenylalanine
2 and *p*-coumaric acid. However, microbial production from cheap substrates such as glucose, glycerol
3 would be more economical.⁴⁰⁹ To achieve resveratrol production in *E. coli* from glucose, various
4 biological strategies were developed, including introduction of the malonate assimilation pathway,
5 enhancement of the malonyl-CoA pool, down-regulation of fatty acid biosynthesis, modification of
6 rate-limiting TAL. The resulting *E. coli* strain proved to be a suitable platform for resveratrol, and the
7 production reached up to 304.5 mg/L from glucose.⁴¹⁹ Alternatively, for an increase of resveratrol
8 and piceatannol, the strategy using modular pathway engineering was applied in *E. coli*. The
9 biosynthesis pathway was divided into three different modules, module I includes polyketide
10 biosynthetic genes, module II includes acetyl-CoA and malonyl-CoA pool-enhancing genes from three
11 different organisms, and module III genes are region specific 3'-hydroxylating enzymes. With the
12 optimized strain, co-expression of the three modules resulted in 127 mg/L piceatannol and the
13 remaining 30 mg/L resveratrol from glucose.¹²⁶ In addition, Summeren and Marienhagen⁴²⁸ examined
14 multiple genes and pathway configurations with *PALs*, *4CLs* and *STSs* from different plants or
15 microorganisms to construct the complete three-step pathway to pinosylvin from L-phenylalanine.
16 3.37 mg/L pinosylvin was produced from glucose by the strain BL21(DE3). With exogenous cerulenin,
17 the pool of malonyl-CoA was enhanced, allowing product titers of 70 mg/L pinosylvin from glucose,
18 further up to 91 mg/L with L-phenylalanine.⁴²⁸ Instead of the expensive cerulenin to enhance the
19 malonyl-CoA, Liang et al.⁴²² used the CRISPRi technique to repress the *fabD* gene involved in fatty
20 acid pathway leading to 47.5 mg/L pinosylvin production in engineered *E. coli* from glycerol. The
21 rational modular design approach was applied for pinosylvin production optimization, leading to the
22 highest pinosylvin titer ever obtained (281 mg/L) from glucose in *E. coli*, in which the overall
23 biosynthetic pathway was divided into three modules, module I with *aroF^{wt}*, *pheA*, *PAL* for cinnamic
24 acid production, the module II with *4CL* and *STS* for pinolysin, module III with anti-
25 *fabF/fabB/eno/adhE/fumC/SucC* sgRNA.⁴²³

26 Glucosylation of stilbenes is known to enhance their water solubility and their biological
27 activity.³²¹ In 2012, Ozaki et al.⁴²⁹ expressed a glucosyltransferase of *Phytolacca americana* (*PaGT3*) in
28 *E. coli*, resulting in the synthesis of two *O*- α -glucoside products, resveratrol-4'-*O*- α -glucoside and
29 resveratrol-3-*O*- α -glucoside. To produce resveratrol glucosides directly from glucose, Choi et al.⁴³⁰
30 constructed an artificial biosynthetic pathway containing *TAL*, *4CL* from *Saccharothrix espanaensis*,
31 *STS* from *A. hypogaea*, and the glucosyltransferase *YjiC* from *Bacillus* in *E. coli*, and then the
32 engineered strain produced resveratrol glucoside derivatives, such as piceid and resveratrol-4'-*O*-
33 glucoside (resveratrolside), from simple carbon sources. The strategy used in this research
34 demonstrates the first harnessing of *E. coli* for *de novo* synthesis of resveratrol glucoside derivatives
35 from a simple sugar medium.⁴³⁰ Recently, a co-culture system of two metabolically engineered *E. coli*
36 populations was employed which comprises an upstream module expressing two enzymes for
37 converting *p*-coumaric acid into resveratrol and a downstream module expressing
38 glucosyltransferase to convert the resveratrol into its glucosylated forms. Upon optimization of the
39 initial inoculum ratio of two *E. coli* populations, 92 mg resveratrol glucosides/L was produced from *p*-
40 coumaric acid.⁴³¹

The methylated stilbene production has attracted much attention for enhancing stilbene bioactivity. Katsuyama et al.¹²² expressed a pinosylvin methyltransferase (*OsPMT*) gene from *Oryza sativa* into the pathway established in *E. coli*, leading to the production of mono- and di-methylated stilbenes. Furthermore, the *OsPMT* gene turned out to be useful in production of unnatural stilbene methyl ethers due to its rather relaxed substrate specificity.¹²² The resveratrol-*O*-methyltransferases (ROMT) from *Vitis riparia* and *Sorghum bicolor* were demonstrated to be active towards resveratrol, giving pinostilbene and pterostilbene.¹²³ Likewise, introduction of a series of codon-optimized *O*-methyltransferase genes from *sorghum* in addition to the resveratrol biosynthetic genes, resulted in the production of various mono, di and trimethylated stilbenes from simple carbon sources.¹²⁴ After that, a resveratrol-*O*-methyltransferase (ROMT) from *Arabidopsis* was also able to catalyze the methylation of resveratrol to pterostilbene. Overexpression of *TAL*, *CCL*, *STS*, and *ROMT* in tyrosine optimized *E. coli* indeed resulted in 3.6-fold increase of pterostilbene, up to 33.6 mg/L in L-methionine-containing media.⁴³²

6.4 Metabolic engineering of stilbenes in non-*E. coli* bacteria

Corynebacterium glutamicum is a well-characterized microorganism that has been used for the production of value-added aromatic compounds, especially amino acids. To produce stilbenes in *C. glutamicum*, Kallscheuer et al.⁴³³ expressed heterologous codon-optimized genes encoding for *4CL* and *STS* and deleted four gene clusters containing 21 genes involved in the catabolism of aromatic compounds. The engineered strain produced up to 158 mg/L of stilbenes when supplemented with *p*-coumaric acid, caffeic acid, and cerulenin. Dereglulation of the shikimate pathway and heterologous expression of *TAL* from *Flavobacterium johnsoniae* resulted in 59 mg/L resveratrol directly from glucose. By optimizing cultivation conditions, 12 mg/L of resveratrol production was achieved without the addition of cerulenin.⁴³⁴ Introduction of an *O*-methyltransferase into a resveratrol-producing *C. glutamicum* strain led to 42 mg/L pterostilbene from *p*-coumaric acid.⁴³⁵

Streptomyces, the largest genus of Actinobacteria, has also been reported as a heterologous host for stilbene production. In order to establish a stilbene producing *Streptomyces* strain, Park et al.⁴³⁶ expressed the stilbene biosynthesis pathway genes in the *S. venezuelae* mutant DHS2001, harboring the *ScCCL* gene from *Streptomyces coelicolor* and the *STS* from *A. hypogaea*. Less than 0.4 mg/L resveratrol was produced by the engineered strain. Additionally, other organisms such as *Aspergillus niger*, *Lactobacillus lactis*, and *Aspergillus oryzae*, have also been used for the bioproduction of resveratrol by incorporating the heterologous pathway genes (*PAL*, *C4H* and *4CL* of *A. thaliana*) and the *STS* of *Rheum tataricum*.⁴³⁷ Recently, oleaginous strains such as *Yarrowia lipolytica* have emerged as powerful host strains for the production of flavonoids and stilbenes.^{438,439} Finally, through protein engineering of stilbene synthase which acts as a type III polyketide synthase, a number of non-natural stilbenes as well as stilbene oligomers have been produced, thus presenting an exciting opportunity for the recombinant production of these molecules.^{88,142,440}

7. Bioproduction of stilbenes by plant cell systems

In comparison with chemical synthesis or genetically engineered bacteria or yeast, plant cell systems including hairy roots (HRs), calli and cell suspension cultures (CSCs), have the potential of

synthesizing very complex compounds that otherwise are very challenging to obtain. In addition, being amenable to genetic transformation, metabolic engineering of plant cell systems has opened new possibilities for exploiting their potential beyond their wild genetic background.⁴⁴¹ Both CSCs and HRs are grown in a liquid medium, and their good performances in large-scale bioreactors have been demonstrated, which makes them good candidates for sustainable industrial process development. As many interesting secondary metabolites are produced in response to stress conditions, elicitation is generally applied as an efficient strategy to promote their production and secretion in plant cell systems,⁴⁴² that is, a highly desirable feature to enable easier downstream processes such as product recovery and purification. Below, we will analyze how these issues apply to the bioproduction of stilbenes and from which new tools and methods they can profit. This topic has already been the matter of some reviews but new and interesting advances have been reached in the last years that deserve a critical and retrospective analysis.^{46,47,443,444}

Unlike flavonoids, stilbenes are not universal metabolites in the plant kingdom, as these have been found in few families of seed plants.⁴⁴⁵ Plant tissue culture methods enable establishment of HRs, calli and CSCs of many plant species, albeit specific protocols must be determined empirically. Right now, plant cell systems have been generated for stilbene bioproduction and studied extensively from *Vitis* spp. and *Arachis* spp. and prospectively from a few other stilbene-producing species such as *Gossypium hirsutum*,⁴⁴⁶ *Morus alba*,⁴⁴⁷ *Ugni molinae*⁴⁴⁸ and *Picea abies*.⁴⁴⁹ Thus, prospection and insight into plant cell systems of still unexplored stilbene-producing species is a field far from being broadly screened and may boost the discovery of new sources of bioactive stilbenes.

7.1. Plant cell systems and strategies

Plant tissues can be stably transformed by infection with *Rhizobium rhizogenes* (formerly *Agrobacterium rhizogenes*).⁴⁵⁰ During the infection process, the bacterium transfers a copy of the T-DNA fragment of the root-inducing Ri plasmid to the plant cell that becomes eventually integrated in the host genome. The genes contained in T-DNA are expressed by the plant machinery leading to the development of highly-branched fast-growing roots named hairy roots (HRs). These roots can develop in a plant growth regulator-free liquid medium and produce high levels of secondary metabolites.⁴⁵¹ HR cultures thus represent a popular method for secondary metabolite production.⁴⁵² On the other hand, disinfected plant explants incubated on sterile solid medium supplemented with an appropriate hormonal balance lead to the development of amorphous cell masses known as callus. Periodic subcultures of the callus in fresh medium gives rise to a cell line that can be maintained indefinitely. Dispersion of the callus in the liquid medium produces cell suspensions (CSCs) that can be conserved as well by periodic subculturing.⁴⁵³

Elicitation is a common strategy applied to plant cell systems to promote secondary metabolite production and secretion and, maybe, the most critical factor in the performance of a given culture system. Elicitors can be physical, (bio)chemical or biological agents that, when present in low amounts, trigger a defense response in the plant cell including the biosynthesis of some defense compounds, namely phytoalexins. Some of them belong to signaling compounds and, among these, jasmonic acid has mainly been used. Jasmonic acid and its derivative methyljasmonate (MeJA) indeed play an integral role in the intracellular signal cascades triggered by elicitor molecules

resulting in the accumulation of secondary metabolites.⁴⁵⁴ Besides, other molecules can be employed for secondary metabolite elicitation in cell or organ cultures. Cyclodextrins, for example, are cyclic oligosaccharides of six to eight glucose residues which derive from starch by the action of the microbial cyclodextrin glucosyl transferase. These are torus-shaped molecules whose internal cavity has hydrophobic character while externally is hydrophilic, thus enabling the formation of water-soluble inclusion complexes with a whole range of apolar ligands including stilbenes.^{455,456} The solubility of β -cyclodextrins is limited but can be enhanced by chemical derivatization, as it is the case for the dimethyl- β -cyclodextrin (DIMEB). Among the elicitors used for stilbene bioproduction in plant cell systems, jasmonates,⁴⁵⁷ UV light,⁴⁵⁸ fungal elicitors,⁴⁵⁹ cyclodextrins⁴⁶⁰ and chitosan⁴⁶¹ have been investigated.

Precursor feeding is also a common strategy in plant bioproduction cell systems to enhance metabolic flow through specific pathways leading to the synthesis of secondary metabolites.⁴⁶² Feeding with ¹³C-labelled phenylalanine has also been studied for the production of isotopically labelled stilbenes.⁴⁵⁷ Metabolic engineering of plant cell systems, in particular overexpression of homologous and heterologous genes, has been performed both in HRs⁴⁶³ and in CSCs⁴⁶⁴ aiming at increasing productivity and diversification of stilbene synthesis as well as overcoming limiting steps in the wild systems.

7.2. Bioproduction of stilbenes in hairy roots

HR cultures have been used for stilbene production in *A. hypogea*⁴⁶⁵, *V. rotundifolia*⁴⁶⁶ and *V. vinifera*.⁴⁶⁷ Generally, HR tissues constitutively accumulate mainly resveratrol and piceid monomers as well as ϵ - and δ -viniferin dimers in the range of tens of mg/g DW with a very low percentage being secreted into the medium. Depending on the elicitation process⁴⁶⁵ and the culture medium⁴⁶⁸, a high accumulation of stilbenes of 352 mg/L has been obtained in *A. hypogea* HRs.⁴⁶⁹ In optimized conditions a production of 750 mg/L of total prenylated stilbenoids after 168h was reported, as well.⁴⁷⁰ Engineering tobacco HRs with genes involved in stilbene biosynthesis or regulation has been carried out in a pioneering study with a limited success.⁴⁷¹ However, this first work opened an interesting and promising way to produce stilbenoids in fast growing HRs.

7.3. Bioproduction of stilbenes in cell suspension cultures

The most promising and widely used plant cell systems for stilbene bioproduction are CSCs. Over calli, CSCs have the advantage of faster growth and better scalability in terms of space requirements, labor force and productivity, *i.e.* cultures in bioreactors, though some studies have shown the ability of either non transformed⁴⁷²⁻⁴⁷⁴ or transformed calli^{245,475,476} for stilbene production.

7.3.1. Cell suspensions: constitutive production and elicitation

V. vinifera cv Gamay Freaux var Teinturier is a pigmented cell line established by the group of Ambid from the pigmented pulp of this grapevine variety and distributed and used worldwide since then. The accumulation of glucosylated stilbenes was first reported in this cell system being *trans*-piceid the most abundant compound, as well as other compounds such as *cis*-piceid and *trans*-astringin.^{477,478}

1 The first experiments describing the role of cyclodextrins as elicitors of stilbene biosynthesis
2 in grapevine cell suspensions were published by the group of Bru et al.⁴⁵⁶ Further, it was shown that
3 both the concentration and the type of the cyclodextrin used affect the level of stilbene
4 accumulation.⁴⁶⁰ Stilbene production indeed increases with 5 to 50 mM DIMEB doses for non-
5 pigmented cv Monastrell cultures grown in the dark stabilizing after four days of incubation at the
6 very high concentration of 14.8 mM (3375 mg/L).⁴⁶⁰ It was shown that modifications in cyclodextrin
7 sugars resembling structural features of oligosaccharides released from plant cell walls during fungal
8 attacks, could be a structural determinant for elicitation.⁴⁶⁰ Response to DIMEB elicitor was found to
9 be species and genotype-dependent as well.⁴⁷⁹ Under equal conditions, suspension cultures of the
10 cross between *V. riparia* and *V. berlandieri*, on one hand, and the *V. amurensis* cell lines, on the
11 other, produced more resveratrol than cultures of *V. vinifera* cv. Pinot Noir or Merzling.⁴⁷⁹ This
12 genotype effect was also demonstrated with Concord grape *V. labrusca* CSCs that exhibited
13 hypersensitivity to the amino acid alanine. This amino acid indeed triggers programmed cell death
14 concomitant with induction of PAL, C4H and STS and production of stilbenes.⁴⁸⁰ Otherwise, chitosan,
15 a major building polysaccharide of fungal cell walls able to induce defense response in grapevine,³⁷²
16 was also proven as an elicitor of stilbene bioproduction in grapevine CSCs of cv Barbera. However, its
17 toxicity to the cultures at a concentration as low as 100mg/L, was responsible for low levels of
18 stilbene induction.⁴⁶¹

19 Direct application of MeJA was reported to induce a high accumulation of piceid in both
20 pigmented cv Gamay Freaux Tenturier and non-pigmented cv Cabernet Sauvignon grapevine CSCs.⁴⁵⁷
21 Both MeJA and JA also triggered higher accumulation of intra- and extracellular *trans*- and *cis*-
22 resveratrol in cv Barbera cultures⁴⁸¹ as well as the accumulation of piceid and ϵ -viniferin in cells of cvs
23 Michele Palieri and Red Globe.⁴⁸² Although jasmonates promoted the expression of *STS* genes in
24 grapevine leaves and CSCs,^{252,483,484} the newly synthesized amounts of stilbenes were rather low.
25 When both DIMEB and MeJA were applied to cv Monastrell cell cultures, expression of the *STS* gene
26 and genes encoding for enzymes of the phenylpropanoid pathway such as *PAL* and *C4H*, increased as
27 compared to their expression with those elicitors used alone.⁴⁸³ It was shown that 50 mM DIMEB, 0.1
28 mM MeJA and 20 g/L sucrose were the optimal conditions for the highest extracellular production of
29 resveratrol.⁴⁸⁵ At the proteomic level, the accumulation of different isoforms of PAL and STS
30 correlated quite well with the levels of resveratrol accumulated under elicitation with MeJA -very
31 low-, DIMEB -high- and MeJA and DIMEB in combination -very high-,⁴⁸⁶ beside extracellular defense
32 proteins.^{485,487}

33 Jasmonyl-isoleucin (Ja-Ile) is an intermediate in the jasmonate signaling pathway that
34 promotes the degradation of JAZ repressor proteins thus allowing the transcription of the JAZ-
35 repressed genes.⁴⁸⁸ The JA-Ile analogue of bacterial origin coronatine (Cor), that can also bind to JAZ
36 proteins, was reported to enhance both the taxane production and taxane biosynthetic gene
37 expression in *Taxus* CSCs.⁴⁸⁹ Thus, Cor was tested as a surrogate of JA-Ile in Monastrell grapevine
38 CSCs for eliciting the bioproduction of stilbenes. Similarly to MeJA, Cor activated the expression of
39 phenylpropanoid and stilbenoid pathway genes but did not induce stilbene secretion to the
40 medium.⁴⁸³ The effect of Cor combined with DIMEB was maximal at 1 μ M as compared to 100 μ M of
41 MeJA and a synergistic effect in resveratrol accumulation in the extracellular medium was also

observed, with a 3.7-fold increase after 168h compared to DIMEB alone.⁴⁸ Although Cor could be used as an elicitor of stilbene bioproduction in plant cell systems instead of MeJA at hundred times less concentration, its cost is about forty thousand times more higher, making it currently not applicable at the industrial level.

Resveratrol added exogenously to grapevine CSCs⁴⁵⁶ as well as to peanut HRs⁴⁶⁹ is rapidly metabolized and disappears from the extracellular medium probably due to its conversion to oligomeric forms through the action of cell wall peroxidases solubilized in the culture medium (see section 2).^{460,487} Also, MeJA elicitation led to a transient accumulation of extracellular resveratrol in CSCs of rootstock 41B (*V. vinifera* cv. Chasselas × *Vitis berlandieri*), then decreasing likely due to subsequent metabolism to oligomeric forms.¹⁴⁷ Such a decrease was not observed upon elicitation with DIMEB⁴⁶⁰ likely due to its ability to form inclusion complexes with resveratrol thus protecting it from further metabolism.^{490,491} Little is known about the mechanisms by which cyclodextrins such as DIMEB trigger a signal pathway that ends up in the production of stilbenes. It has been shown that Ca⁺² mobilization from extra- and intracellular compartments, production of H₂O₂ and NO, involvement of MAPKs and Tyr phosphatases are essential events for the accumulation of resveratrol upon DIMEB elicitation.⁴⁹² Use of MeJA in addition to DIMEB alleviates the effect of extracellular Ca⁺² blockers, MAPKs inhibitors and NO scavengers suggesting that there are some events specifically triggered by DIMEB and others which are enhanced by MeJA. In leaves of the Chinese wild grapevine *V. quinquangularis*, a Raf-like *MAPKKK* gene, *VqMAPKKK38*, was shown to positively regulate *STS* transcription and stilbene accumulation probably by activation of the transcription factor MYB14 (see section 3). In turn, both H₂O₂ and Ca⁺² influxes as well as treatments with hormones such as salicylic acid or MeJA, activated *VqMAPKKK38* expression and stilbene biosynthesis.²⁶²

Optimization of stilbene production using experimental designs can help to find optimal ranges of several production medium components with a minimal number of experiments. Using a full factorial experimental design for evaluating the effect of biomass, MeJA and sucrose concentrations, stilbene production was found as optimal in the following conditions: 5.6 g biomass DW/L, 0.7 mM MeJA and no extra sucrose added in *V. labrusca* CSCs achieving 206 mg/L resveratrol and 124 mg/L pallidol as major stilbenes.⁴⁹³ Furthermore, when using stilbene adsorbents such as the hydrophobic resins XAD or DIMEB, the stilbene production was significantly increased with drastic changes in the stilbene profiles in the medium. In case of XAD, pallidol was more abundant reaching 900 mg/L, followed by ε-viniferin 500 mg/L, resveratrol 380 mg/L and δ-viniferin 198 mg/L; in case of DIMEB, the major stilbenes were resveratrol 6100 mg/L, followed by δ-viniferin 537 mg/L, ε-viniferin 231mg/L and 116 mg/L pallidol, each compound peaking at different incubation times.⁴⁹³

7.3.2. Cell suspensions: precursor feeding

The ability of grapevine CSCs to convert stilbene precursors such as phenylalanine has been utilized to produce isotopically labelled stilbenes that constitute valuable tools for *in vivo* metabolic studies. In MeJA-elicited *V. vinifera* cv. Cabernet Sauvignon cultures, a maximal production of ca 1 mmol per L of culture of piceid with a 60% ¹³C enrichment was achieved by adjusting ¹³C-Phe concentrations and addition patterns as an excess of this precursor was observed to be detrimental to cell growth and piceid accumulation.⁴⁵⁷ Since phenylalanine is the precursor of most polyphenols,

labeling of other end-products including catechins and anthocyanins also occurred to a great extent in the pigmented Gamay CSCs.^{494,495} Elicitation that opens up the stilbene metabolic gate, would be expected to put in place a strong competition for the precursor *p*-coumaroyl CoA. However, treatment with MeJA and fungal elicitors revealed that such a competition only weakly takes place, the metabolic flow being channelled through the flavonoid pathway.⁴⁵⁹ Feeding ¹³C-Phe was then used to produce high titers of 2666.7 mg/L ¹³C-labelled resveratrol,⁴⁹⁶ however the enrichment rates achieved of 20-35% were significantly lower than those reported for labelled piceid upon MeJA elicitation.⁴⁵⁷

7.3.3. Cell suspensions: metabolic engineering

Because key genes of stilbene biosynthesis have a very low expression in non-senescent tissues and are inducible by biotic and abiotic stresses in grapevine,³³ and also in most of plant cell systems, a way to achieve a constitutive production of these compounds is to metabolically engineer the cell lines with genes under control of constitutive promoters such as CaMV 35S (see section 5). Optimization of a stable transformation protocol for grapevine cv. Gamay and Monastrell calli⁴⁶⁴ has allowed the efficient overexpression of resveratrol-modifying enzymes including hydroxylation by a human cytochrome P450 (*HsCYP1B1*) and methylation by the *V. vinifera* resveratrol-*O*-methyl transferase (*VvROMT*). Upon elicitation with DIMEB and MeJA, the synthesis of large amounts of resveratrol was accompanied by the production of the more potent antitumoral stilbenes, piceatannol⁴⁹⁷ in the *HsCYP1B1*-transformed lines, and pterostilbene⁴⁹⁸ in the *VvROMT*-transformed lines.⁵⁰ Because production of pterostilbene was quite low, CSCs were further transformed with an orcinol-*O*-methyl transferase from *Rosa hybrida* (*RhOoMT*), an enzyme that shares a 69.5% similarity with *VvROMT* and whose substrate, orcinol, contains a *m*-diphenol moiety comparable to resveratrol. The resulting transformed lines were treated with elicitors in the same way then achieving a higher production of pterostilbene.⁴⁹⁹ Titrers recovered were: 20 mg/L piceatannol, 16 mg/L pterostilbene as well as the simultaneous production of resveratrol between 1500-3000 mg/L, depending on the cell line.

Overexpression of *VvSTS* genes has also been proposed as a strategy to increase the bioproduction of resveratrol in CSCs. Overexpression of the *STS* gene in grapevine cv Monastrell had no significant negative effect on culture growth or cell viability and led to a maximal resveratrol production under MeJA and CD elicitation of 1458 mg/L in the transformed lines.⁵⁰⁰ Transformed Monastrell cell lines have been shown to achieve a two-fold increase in stilbene production under MeJA and DIMEB elicitation⁴⁹⁹ compared to the wild-type cell lines.⁴⁸³ Since the substrates for *STS* are available in all plants, the potential of metabolically engineered *Sylibum marianum* cultures for resveratrol production was explored through the overexpression of the *VvSTS3* gene. Transformation led to an extracellular accumulation of *trans*-resveratrol up to 12 mg/L under elicitation with DIMEB in addition to an accumulation of the silymarin and coniferyl alcohols as in the wild-type lines.⁵⁰¹

Transformation of the grapevine pigmented line cv. Gamay Red with a feedback-insensitive bacterial form of the 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase enzyme (AroG*), which catalyzes the first step of the shikimate pathway (see Figure S1), accumulated up to 20-fold higher levels of resveratrol and also higher levels of metabolites from the shikimate,

phenylpropanoid and flavonoid pathways suggesting that precursor availability can be a major limiting factor for polyphenol accumulation.⁵⁰² In addition to stilbene biosynthesis, transport to the medium is a key issue that can be considered for stilbene production engineering in CSCs, as well. Although no specific resveratrol transporters have been characterized to date, proteomic^{486,503} and transcriptomic experiments⁵⁰⁴ on DIMEB and MeJA-elicited CSCs revealed that the glutathione-S-transferase VvGSTU-2, a multidrug resistance-associated type protein, strongly co-expresses with PAL and STS isoforms. As paralogues of GST are involved in the vacuolar accumulation of anthocyanins, the *GSTU-2* candidate was overexpressed in cv Gamay CSCs that accumulate intracellular piceid constitutively. Transformed cultures assisted by adsorbents in the medium such as β -cyclodextrin or polyethylene glycol accumulated 44- and 65-fold respectively more extracellular resveratrol than the wild-type lines thus providing strong evidence of the involvement of GST in the extracellular accumulation of resveratrol.⁵⁰³ The mechanism of this transport process remains unknown but as this protein has been shown to act as ligandins in addition to its enzymatic activity carrying for example, compounds like anthocyanins,⁵⁰⁵ the proposed role of resveratrol carrier for VvGSTU-2 was suggested.

Generation of stably transformed calli or CSCs can last for at least six months of which the callus selection is the most intensive labor phase. An alternative way was carried out by the quick generation of tobacco HRs using *R. rhizogenes* transformed with binary plasmids harboring the genes of interest. After faster selection of the transformed HR lines by PCR screening, calli and CSCs were generated by adding plant growth regulators to the solid growth medium thus inducing dedifferentiation and allowing the establishment of calli and subsequently CSCs.⁴⁶³ Both tobacco HRs and CSCs generated thereof and transformed with *HsCYP1B1* were able to convert resveratrol into piceatannol at a faster rate than non-transformed ones. A production of 7 and 1 mg/L of piceatannol was achieved respectively in transformed HRs and CSCs. Incorporation of cassettes also including *STS* might enable this system to readily produce stilbenes using cheaper precursors such as sucrose or phenylalanine.⁴⁶³

7.4. Scale-up of resveratrol bioproduction by grapevine cells

Upscaling the bioproduction of active compounds such as stilbenes using plant CSCs from shaken flasks to a commercial scale involves the essential step of the transfer of the process to bioreactors. Cultivation of plant CSCs in volumes as large as 75,000 L can be performed by industries. As the performances of the cultures are species-, variety- and even cell line-dependent, optimization of the culture systems has to be resolved in a case-by-case manner. Several bioreactor designs including stirred tanks, airlifts, bubble columns and disposable concepts have been shown to cope well with shear sensitivity and have successfully been used to grow grapevine CSCs.

Table 2 summarizes the grapevine biomass concentration and productivity achieved among the different studies carried out in bioreactors. A comparison between two bioreactor designs, stirred tanks and V-shaped bubble columns, shows that the growth rate constant μ may not significantly vary with sucrose concentration and only slightly among bioreactors. On the other hand, the maximum biomass production and the productivity greatly depend on the initial sucrose concentration and could be limited by the oxygen supply for high biomass concentrations.⁵⁰⁶

Although the culture of grapevine cells in bioreactors has been successful, yield and productivity differences are in part due to variations in shear sensitivity of the cell lines. *V. labrusca* CSCs endured the shear conditions in laboratory and pilot scale stirred tank bioreactors under similar aeration and agitation conditions.^{51,493} However, the rootstock 41B cell line only tolerated very low aeration flows and agitation rates¹⁴⁷ and the Barbera cell line underwent a decrease of cell biomass of 30%.⁵⁰⁷ Considering the heterogeneity between studies, the best parameter to choice for establishing comparisons among experiments would be the maximal cell concentration achieved. The highest concentration of 884 g FW/L was recorded in a disposable bag wave bioreactor (40 g/L.day⁻¹).⁵⁰⁸ In stirred tank bioreactors, a higher biomass concentration (from 250-400 g/L) is generally achieved compared to bubble columns (130-150 g/L) (Table 2), likely due to a more effective mass transfer rate by the combination of stirring and aeration. In stirred tank type bioreactors, biomass productivities as high as 32 g/L.day⁻¹ were reported.⁴⁹⁵ Operating in feed-batch mode, *V. labrusca* CSCs have been scaled up from 1 L flasks to laboratory and pilot scale bioreactors of up to 20 L operating volume. Maximal biomass concentration and productivity were in the range of those achieved for *V. vinifera* lines in laboratory scale stirred tank bioreactors, thus showing the scalability of the cultures.^{51,493,509}

Table 3 summarizes the results obtained with regard to stilbene production in bioreactors using grapevine CSCs. Early works focused on the production of ¹³C bio-labeled polyphenolics, including stilbenes, through a labeled precursor-feeding approach under optimized growth conditions leading only to intracellular piceid production⁴⁹⁵ as occurred when non-labeled precursors were added.⁵¹⁰ Subsequent studies have utilized elicitors, in particular chitosan,⁵⁰⁷ MeJA,¹⁴⁷ β -cyclodextrin and MeJA⁵¹ and DIMEB alone or combined with MeJA,^{493,506} with the aim of enhancing stilbene production and promoting stilbene extracellular accumulation.

Resveratrol production is often reported as grams per liter of culture but the biomass concentration has a strong influence on the overall stilbene production. Thus, a more appropriate variable to compare across different studies is the specific production (milligrams of compound per gram biomass) referred to either fresh or dry cell weight. Elicitation with chitosan or MeJA led to a similar yield above 1 mg/g FW, slightly improving the yield in shaken flasks. However, while chitosan led to a mixture of intra- and extracellular resveratrol monomeric isomers in Barbera cell lines, elicitation of the rootstock 41B with MeJA mainly produced *trans*-resveratrol in the medium as well as the dimer ϵ -viniferin (*ca* 3%) and trace amounts of piceatannol. Upscaling of production with the cv Gamay under elicitation with DIMEB in stirred tanks and in V-shaped bubble column bioreactors led to resveratrol yields of 3 and 2.2 mg/g FW, respectively and 13.5 mg/g FW in bioreactors when the elicitation combined DIMEB and MeJA.⁵⁰⁶ Whereas, starting a batch culture of 1 or 2 liters can be performed with a whole inoculum previously grown in shaken flasks and then incubated with the complete elicitation medium though the same strategy is unfeasible when scaling to tens or hundreds of liters of culture. This requires a previous biomass growth phase in the bioreactor used for production followed by addition of the elicitors when the appropriate biomass density is obtained. Initial operations in stirred tank bioreactors with *V. labrusca* CSCs and continuous feeding with the fresh medium allow to achieve cell densities between 170-230 g FW/L in a final volume of up to 20 L after *ca.* 11 days, moment at which MeJA elicitation started. As a result, stilbenes

accumulated in the medium peaking around 7 days from elicitation, the composition and yield of which was shown very dependent of the type of the elicitor used.⁴⁹³ A final yield of 14.3 mg/g FW resveratrol upon addition of MeJA was then achieved.

To address the problem of the addition of the complete elicitation medium (DIMEB + MeJA) to the whole inoculum, a bubble column bioreactor that allows a complete exchange of the culture medium was designed and built at a 7 L demonstration scale.⁵¹¹ As the old medium (*i.e.* for growth or elicitation) can be completely removed and replaced by the fresh medium many times, a consecutive number of batches of either growth or elicitation were performed using cv Gamay CSCs. Cells used up to three times for production led to *ca* 11 mg resveratrol/g FW per batch on average, thus producing 32.7 mg/g FW during a one stage production period of 13 days and 33.3 mg/g FW during eight days of growth phase followed by 16 days of production.⁵⁰⁶ Although this level of stilbene bioproduction, particularly resveratrol, is one of the highest ever reached for a bioactive compound, the bottleneck that remains for its commercial exploitation is the high cost of cyclodextrins. Thus, alternative elicitors and/or solubilizers suitable for use at large scale that can compete in performance and cost with cyclodextrins would overcome that hurdle and enable a sustainable bioproduction of stilbenes by plant cell systems. In that sense, cyclodextrin polymers coated with magnetic nanoparticles have recently been investigated as easily recoverable and reusable elicitors in Monastrell grapevine cell cultures for resveratrol bioproduction as an alternative to soluble cyclodextrins.⁵¹² It is expected that such a strategy would reduce the production costs.

8. Conclusions and perspectives

Many aspects of stilbene chemistry, biochemistry and metabolism as well as stilbene functions in plants, have been depicted in this review. Engineering methods for introducing stilbene biosynthetic pathways in plants and microorganisms and stilbene bioproduction by plant cell systems were also discussed.

The stilbene chemistry, namely that of resveratrol-based oligomers, appears to be linked to the condensation of various resveratrol phenoxyl radicals leading to very reactive *para*-quinonic systems whose rearrangement through oxa-Michael additions and/or Friedel-Craft reactions yields very diverse structures (section 2). In addition to the continuous identification of new oligomeric stilbenes, a question that remains that should arouse some interest in the coming years, is the deciphering of the mechanisms which generate stereoconditions controlling the formation of optically active stilbenes.²⁹ To explain it, the role of putative dirigent proteins as those previously described by Pickel et al. for the laccase-mediated coupling of phenols in lignan formation^{172,513}, has been suggested for the regio and stereoselective coupling of the produced resveratrol radical entities (Figure 3).²⁹ Similar DIRs have also recently been described in fungi, for example during the phenol-oxidative coupling for the biosynthesis of the cytotoxic and antibacterial viriditoxin in *Paecilomyces variotii*.⁵¹⁴ Thus, characterizing such proteins would constitute an exciting challenge for stilbene chemistry.

Even if stilbenes have been defined as phytoalexins, their antifungal activity is low especially when addressing hydroxystilbenes (section 4). Future works should thus encompass the production of more active derivatives based on resveratrol and oligomeric structures. Significant progress has

1 been made in the programmable and controlled chemical synthesis at the gram scale of some
2 stilbene oligomers such as ampelopsin H and carasiphenol C by Snyder's group.⁴² This remarkable
3 work lays the groundwork for the future synthesis of other stilbenes in quantities allowing
4 evaluation of their biological activity.

5 The chemical spaces of stilbenes could also be increased through use of both microbial
6 engineering and structure-guided mutagenesis of the stilbene synthase (section 6). It has indeed
7 been reported that stilbenes containing halogens, a furan or a thiophen ring show interesting
8 biological activities and have already been produced through microbial engineering using
9 recombinant *E. coli*.^{89,90} Various unnatural stilbenes have also been obtained using structure-guided
10 mutagenesis of STS.^{88,440}

11 Knowledge on the control mechanisms involved in stilbene biosynthetic pathways has made
12 great progress as many transcription factors regulating stilbene synthase have been identified so far
13 (section 3).³⁷⁶ The recent characterization of transcriptional factors such as VaMyb1 and WRKY8,^{261,265}
14 which negatively regulate stilbene biosynthesis in grapevine, can provide an explanation of the fact
15 that, in some instances, STS overexpression does not lead to the expected overproduction of
16 stilbenes in transgenic plants. This raises the question of introducing not only the *STS* gene upon
17 genetic transfer experiments but also working on the engineering of the transcription factors which
18 down regulate stilbene biosynthesis. Silencing of these negative regulatory loops in plants could thus
19 afford new insights in plant engineering with stilbene synthase genes (section 5).

20 Metabolic engineering of stilbene biosynthetic pathways in microbes has yielded impressive
21 results with regard to the production of resveratrol and its derivatives (section 6). In several
22 publications, hundred milligrams or grams per liter titers have been reported. However, Li et al.³⁸⁷
23 have calculated that the resveratrol production yield actually performed from glucose in a
24 transformed yeast strain was only 2.5% of the theoretical yield. There is thus a need for improved
25 strategies in the field of stilbene engineering in microbial cells. Malonyl-CoA is a crucial node at the
26 intersection of the stilbene and flavonoid pathways⁵¹⁵ and fatty acid biosynthesis. Regulating both
27 the upstream and downstream pathways of this key metabolite will constitute a future option for the
28 metabolic engineering of stilbene pathways in bacteria. In that sense, the development of biosensors
29 for the control of malonyl-CoA production, on one hand, and its sink pathway (fatty acid
30 biosynthesis) on the other, will constitute an interesting approach to redirect the metabolism
31 towards this compound as previously reported in the yield optimization of fatty acid biosynthesis in
32 *E. coli*.⁵¹⁶ Finally, other recent techniques such as the Operon-PLICing method, which allows the
33 building of operons integrating multiple target genes under the control of a single promoter, would
34 be perfectly suited for engineering complex pathways such as the stilbene pathway.⁵¹⁷

35 Engineering of stilbene pathways in microbes (section 6) as well as stilbene bioproduction by
36 plant cell cultures (section 7) have demonstrated the possibility of producing resveratrol in the gram
37 order with these systems. The main challenge in this domain is to up-scale resveratrol and stilbene
38 production at the industrial level. Another problem is the purification of the obtained stilbenes in
39 complex mixtures. Combining centrifugal partition chromatography with subsequent identification of
40 the compounds by ¹³C NMR dereplication method may address interesting solutions.^{51,148,518,519}

Legends of the figures:

Figure 1: General scheme illustrating stilbene biosynthetic pathways from phenylalanine.

Abbreviations used: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; CL/4CL, cinnamoyl/4-cinnamoyl-CoA ligases; STS, stilbene synthase; PS, pinosylvin synthase; CHS, chalcone synthase; ROMT, resveratrol-O-methyltransferase; POMT, pinosylvin-O-methyltransferase; GT, glucosyltransferases; UDPG, UDP-Glucose; PER, peroxidases; ACC, acetyl-CoA carboxylase complex

Figure 2: Structures of characteristic stilbene monomers. Hydroxystilbenes, resveratrol, piceatannol and pinosylvin; stilbene glucosides, piceid, astringin; methylated stilbenes, pterostilbene; isorhapontigenin, isorhapontin, trimethoxystilbene; isoprenylated stilbenes; arachidin-1, arachidin-2 and arachidin-3; unnatural stilbenes, 4,4'-dihydroxystilbene; hexahydroxystilbene, 3,4,5-trimethoxy- β -(2-furyl)-styrene, 4-methoxy- β -(2-furyl)-styrene and 3,5-dimethoxy- β -(2-furyl)-styrene

Figure 3: Different modes of coupling of phenoxyl resveratrol radicals. According to different modes of coupling, 3-8', 8-8', 8-10' and 8-12', various dimers can be obtained as illustrated by the formation of δ -viniferin (3-8'), pallidol (8-8'), ampelopsin F (8-10') and gnetin C (8-12') (according to Stephenson's group²⁹)

Figure 4: Examples of stilbene dimers resulting from 8-8' condensations. Condensation of two phenoxyl radicals C leads to the formation of two hypothetical intermediates yielding, respectively, pallidol and restrytisol A, quadrangularin A and leachianols F/G (according to Stephenson's group²⁹)

Figure 5: Examples of stilbene dimers resulting from 8-10' condensations. Condensation of the phenoxyl radicals D + C leads to the formation of hypothetical intermediates yielding, respectively, (+)- ϵ -viniferin and (+)-ampelopsin B, (+)-ampelopsin F and (-)-ampelopsin D (according to Stephenson's group²⁹)

Figure 6: Possible transformation pathways from (-)- ϵ -viniferin to various oxidized 8-10' stilbene dimers (balanocarpol, malibatol and hopeahainanphenol) (according to Stephenson's group²⁹)

Figure 7: Examples of stilbene trimers resulting from 10-8' or 8-10' condensations. 10-8' condensations of phenoxyl radicals of resveratrol and ϵ -viniferin, yields respectively gnetin H and miyabenol C (according to Stephenson's group²⁹)

Figure 8: Examples of stilbene tetramers resulting from the 8-8' condensation of ϵ -viniferin. Friedel-Crafts cyclization reactions involving carbons 14b and 7c as well as carbons 14c and 7b lead to ampelopsin H. Viniferol A, is obtained from a cyclization involving carbons 14b and 7c, carbons 10d and 7b. Hopeaphenol results from Friedel-Crafts reactions between carbons 10d and 7c, carbons 10a and 8b, respectively (according to Stephenson's group²⁹ and He's group¹³⁸)

Figure 9: Fungal metabolism of resveratrol by *Botrytis cinerea* and *Neurospora crassa*. Metabolism of resveratrol by the *B. cinerea* laccase yields a number of dimers including δ -viniferin, restrytisols A and B, restrytisol C, pallidol and leachianols F/G (according to Cichewicz et al.¹⁵¹) Lower part of the figure: metabolism of resveratrol by *N. crassa* by the carotenoid cleavage oxygenase CAO-1, leading to 3,5-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde (according to Diaz-Sanchez et al.³²³)

Figure 10: Fungal metabolism of astringin by *Ceratocystis polonica*. Fungal catabolism involves deglycosylation, dimerization and oxidation reactions leading respectively to astringin or piceatannol dimers, on one hand, and lactones on the other hand (according to Hammerbacher et al.³⁴)

Figure 11: Metabolic engineering of stilbene biosynthetic pathways and subsequent metabolism in yeast. Abbreviations used: *ARo4* and *ARo7*, insensitive alleles of DAHP synthase and chorismate mutase, respectively; *PAL*, phenylalanine ammonia lyase; *TAL*, tyrosine ammonia lyase; *C4H*, cinnamate-4-hydroxylase; *CPR*, cytochrome P450 reductase; *4CL*, 4-cinnamoyl-CoA ligases; *STS*,

1 stilbene synthase; *ROMT*, resveratrol-*O*-methyltransferase; *GT*, glucosyltransferases; *ACC*, acetyl-CoA
2 carboxylase complex

3 **Figure S1: Biosynthetic pathways to aromatic amino acids and malonyl-CoA from glucose.**

4 Abbreviations used: DAHP, 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate; *ACC*, acetyl-CoA
5 carboxylase complex, *CM1*, chorismate mutase 1; *PPA-AT*, prephenate aminotransferase; *ADT*,
6 arogenate dehydratase; *ADH*, arogenate dehydrogenase

7 **Figure S2: Putative relationships between 8-8' dimeric naturally-occurring stilbenes (according to**
8 **Stephenson's group²⁹)**

9 **Figure S3: Examples of stilbene tetramers resulting from the 3-8' condensation of *e*-viniferin** The
10 two tetramers, vitisin A and vitisin B isolated from grapevine root extracts result from the 3-8'
11 coupling of two oxidized molecules of (-)-*ε*-viniferin (according to Stephenson's group²⁹)

12 **Figure S4: Hypothetical mechanism for the formation of a stilbene heptamer.** The stilbene
13 heptamer pauciflorol D is formed by condensation between the resveratrol 8-10' tetramer,
14 vaticaphenol A, and an 8-8' quinone trimer formed by the coupling of three resveratrol
15 hydroxyphenyl radicals C (according to Stephenson's group²⁹)

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Figure 1

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2 Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Metabolism by *Botrytis cinerea*

Metabolism by *Neurospora crassa*

Figure 9

Metabolism by *Ceratocystis polonica*

Figure 10

Figure 11

Table 1 Plant transformation with stilbene synthase genes and biological effects					
Plant used for transformation	Gene(s) transferred	Promoter	Stilbenes produced	Biological effect	References
Tobacco	<i>STS</i> gene from <i>Arachis hypogea</i>	Stress-induced promoter	Resveratrol (0.05 µg/g Cell suspensions)	Not described	340
Tobacco	<i>Vst 1</i> and <i>Vst2</i> genes from <i>Vitis vinifera</i>	<i>Vst1</i> (own inducible promoter)	Resveratrol (400 µg/g FW)	Resistance to <i>Botrytis cinerea</i> but not in all lines	39
Tobacco	<i>Vst 1</i> gene from <i>V. vinifera</i>	CaMV35S or tapetum-specific, <i>tap1</i>	50-70 µg/g FW in male fertile and 290 µg/g FW in male sterile leaves	Alteration of flower morphology, male sterility	362
Rice	<i>Vst 1</i> gene from <i>V. vinifera</i>	<i>Vst1</i> (own inducible promoter)	Not given	Reduction of leaf symptoms caused by <i>Pyricularia oryzae</i>	359
Tomato	<i>Vst 1</i> and <i>Vst2</i> genes from <i>V. vinifera</i>	<i>Vst1</i> (own inducible promoter)	Resveratrol (12-602 µg/g FW) depending on the pathogen	Resistance to <i>Phytophthora infestans</i> . No resistance to <i>B. cinerea</i> and <i>Alternaria solani</i>	363
Barley and wheat	<i>Vst 1</i> gene from <i>V. vinifera</i>	<i>Vst1</i> + 4-fold CaMV35S enhancer	Resveratrol (not determined)	Enhanced resistance to <i>B. cinerea</i> in barley	346
Wheat	Chimeric <i>STS</i> gene from <i>V. vinifera</i>	Ubiquitin promoter from maize	Not given	Not described	347

Alfalfa	<i>AhRS</i> gene from <i>A. hypogea</i>	CaMV35S	Piceid (20 µg/g FW)	Resistance to <i>Phoma medicaginis</i>	342
Kiwifruit	<i>pSV25</i> gene from <i>V. vinifera</i> or <i>pLAB</i> from <i>V. labrusca</i> or <i>pRIP</i> from <i>V. riparia</i>	CaMV35S	Piceid (182 µg/g FW young leaves and 20 µg/g FW old leaves)	No increased resistance to <i>B. cinerea</i>	354
Wheat	<i>Vst 1</i> gene from <i>V. vinifera</i>	Vst1 + 4-fold CaMV35S enhancer	Not given	Variable resistance to <i>Plasmopara viticola</i>	348
Grapevine	<i>Vst 1</i> gene from <i>V. vinifera</i>	Pathogen inducible PR 10 from alfalfa	Resveratrol (2000 µg/g DW after <i>B. cinerea</i> inoculation)	Resistance to <i>B. cinerea</i> in 40% of leaves	351
Apple	<i>Vst 1</i> gene from <i>V. vinifera</i> <i>PGIP</i> gene from kiwifruit	Vst1 (own pathogen inducible promoter)	Not given	Not determined	343
White poplar	<i>StSy</i> gene from <i>V. vinifera</i>	CaMV35S	Piceid (615 µg/g FW)	No increased resistance to <i>Melampsora pulcherrima</i>	369
Aspen	Pinosylvin synthase <i>PS</i> gene from <i>Pinus sylvestris</i>	CaMV35S	Not detected	1/3 line showed increased resistance to <i>Phenillus tremulae</i>	285
Papaya	<i>Vst 1</i> gene from <i>V. vinifera</i>	Vst1 (own pathogen inducible promoter)	Resveratrol glucoside (54 µg/g FW)	Increased resistance to <i>Phytophthora palmivora</i>	357
Tomato	<i>StSy</i> gene from <i>V. vinifera</i>	CaMV35S	Resveratrol (53 µg/g FW) in red stage fruits	Increased antioxidant activity of fruits	364
Oilseed rape	<i>Vst 1</i> gene from <i>V. vinifera</i> + Silencing of the <i>Bn SGT1</i> gene	Seed-specific napin promoter	Resveratrol glucoside (361-616 µg/g seeds)	Decrease of the sinapate ester content and increased forage value	356
Chinese digitalis	<i>AhRS3</i> gene from <i>A. hypogea</i>	CaMV35S	Resveratrol glucoside (22-116 µg/g leaves)	High resistance to <i>Fusarium oxysporum</i>	350

Bread wheat	<i>Vst 1</i> and <i>Vst2</i> genes from <i>Vitis vinifera</i> <i>Pss</i> pinosylvin synthase gene from <i>P. sylvestris</i>	<i>Vst1</i> and <i>Vst2</i> own inducible promoters + 4-fold CaMV35S enhancer	35-190 µg/g FW unknown resveratrol or pinosylvin analogs	Increased resistance to <i>Puccinia recondita</i> and <i>Septoria nodorum</i>	349
Arabidopsis	<i>SbSTS1</i> gene from <i>Sorghum bicolor</i>	CaMV35S	-	First example of a <i>STS</i> gene from monocot	177
Lettuce	<i>STS</i> gene from <i>Parthenocissus henryana</i>	CaMV35S	Resveratrol (56 µg/g FW)	Anticancer activity in HeLa cells	355
Tomato	<i>StSy</i> gene from <i>V. vinifera</i>	CaMV35S	Not given	Increased antiradical activity of fruits	365
Apple	<i>Vst 1</i> gene from <i>V. vinifera</i>	<i>Vst1</i> own inducible promoter	Piceid (23-62 µg/g FW after UV-irradiation)	Not determined	344
Arabidopsis	<i>SbSTS1</i> gene from <i>Sorghum bicolor</i>	CaMV35S	Cis-piceid (580 µg/g FW)	Not determined	345
Pea	<i>Vst 1</i> gene from <i>V. vinifera</i>	<i>Vst1</i> (own pathogen inducible promoter)	Resveratrol (0.5 to 5 µg/g FW)	Not determined	358
Tomato	<i>StSy</i> gene from <i>V. vinifera</i>	CaMV35S	Resveratrol (48 µg/g peel) Piceid (126 µg/g peel)	Decrease in naringenin, rutin and chlorogenate	85
Hop	<i>Vst 1</i> gene from <i>V. vinifera</i>	CaMV35S	Piceid (560 µg/g FW in hop cones)	Not determined	353
Grapevine	<i>STS</i> gene from <i>V. pseudoreticulata</i>	CaMV35S	Resveratrol (2.6 µg/g FW)	Not determined	352
Strawberry	<i>NS-Vitis 3</i> gene from <i>V. riparia</i>	CaMV35s + flower-specific promoter (<i>fil1</i>) from <i>Antirrhium majus</i>	Resveratrol or stilbenes (not detected)	Increased sensibility to <i>B. cinerea</i> , down-regulation of CHS	86

Tomato	<i>StSy</i> gene from <i>V. vinifera</i>	Fruit specific promoter TomLoxB or CaMV35S	Piceid (126 µg/g FW skin) under CaMV35S Piceid (6 µg/g FW skin) under TomLoxB	Increased antioxidant activity of fruits	366
Rice	<i>AhSTS1</i> gene from <i>A. hypogea</i>	Ubiquitin 1 (Ubi 1) promoter from maize	Piceid (1-174 µg/g FW leaves)	Anti-metabolic syndrome activity	360
Arabidopsis	<i>STS</i> gene from <i>Polygonum cuspidatum</i>	CaMV35S	Piceid (93-184 µg/g FW leaves) Piceid (15-36 µg/g FW seeds)	Decrease of lesion diameter on leaves caused by <i>Colletotrichum higginsianum</i>	87
Purple sweet tomato	<i>RS</i> gene from <i>A. hypogea</i>	CaMV35S	Resveratrol glucoside (52- 340 µg/g DW)	Not determined	367
Tobacco and Arabidopsis	<i>AhSTS3</i> gene from <i>A. hypogea</i> + <i>SbOMT3</i> gene from <i>S. bicolor</i>	CaMV35S	Pterostilbene (8 to 52 µg/g FW) for Arabidopsis	Not determined	109
Soybean	<i>AhRS3</i> gene from <i>A. hypogea</i> + <i>ROMT</i> gene from <i>V. vinifera</i>	Cassava Vein Mosaic Virus (Cs VMV) promoter	Resveratrol (30 to 350 µg/g FW), pterostilbene (5-8 µg/g FW)	Increased resistance to <i>Rhizoctonia solani</i>	368
Rice	<i>PNRS1</i> gene from <i>A. hypogea</i>	Ubiquitin 1 (Ubi 1) promoter from maize	Resveratrol (3 µg/g DW seeds)	Protection from UV or dark-induced senescence	361

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Table 2 Production of <i>Vitis</i> sp. cell biomass in bioreactors							
Bioreactor design/ operation mode	Working volume (L)	Inoculum % (FW/100mL)	Cell line/[sucrose] (g/L)	Aeration(vvm)/ agitation(rpm)	Max. biomass concentration (g FW/L)	Biomass productivity (g FW/L/d)	Reference
Stirred tank/ batch	15	5.75 ^a	Gamay Fréaux/60	0.2/100	269	10	510
Stirred tank/ batch	2	11.5 ^a	Gamay Fréaux/50	0.075-0.15/75	259	32	495
Stirred tank/ batch	0.8	2	Barbera/10	0.2/100	40	2.85	507
Stirred tank/ batch	2	7.5 ^b	Rootstock 41B/30	0.025/50	273	18	147
Stirred tank/ batch	1.1	1 ^b	Gamay/30	2/80	403	23	506
Stirred tank/ feedbatch	10	9.5	V. labrusca Concord/30	0.025/50	110	5.2	51
Stirred tank/ feedbatch	5	8 ^c	V. labrusca Concord/30	0.01-0.4/100	317 ^c	16.1 ^c	509
Stirred tank/ feedbatch	5 and 20	9.5 ^c	V. labrusca Concord/30	0.15/100 (5L) 0.15/50(20L)	224 ^c (7/DW) (5L) 324 ^c (10.2/DW) (20L)	15.8 ^c 23.7 ^c	493
Wave	1	--	--	--	884	40	508
Bubble column V- shaped	1.1	11 ^b	Gamay/30	0.95/--	150	10	506
Bubble column cylindrical	5.8	2.23	Gamay/20	0.3-0.6/--	137.8	4.8	506

2 ^aEstimated considering the dilution factor of flasks producing 460g/L biomass. ^b Estimated considering final biomass and fold increase. ^cEstimated considering that an inoculum of 15g DW/L⁴⁹³
3 is equivalent to an inoculum of 475g FW/L,⁵¹ thus conversion factor is DW=0.0315.FW. When no explicit data were stated in papers, these were calculated after the graphical material
4 contained in them.

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Table 3 Resveratrol bioproduction by grapevine cell suspensions in bioreactors							
Bioreactor design	Process mode	Working volume (L)	Inoculum % (FW/100mL)	Cell line/[sucrose] (g/L)	Elicitor	Maximum Stilbenoid yield (mg/g FW) and location	Reference
Stirred tank	Batch, one-stage	15	5.75 ^a	Gamay Fréaux/60	none	piceid 0.11 intracellular	510
Stirred tank	Batch, one-stage	2	11.5 ^a	Gamay Fréaux/50	none	piceid 0.54-0.70 intracellular	495
Stirred tank	Fed-Batch, two-stages	0.8	4	Barbera/30	chitosan	stilbenoid monomers mix. 1.2 extra and intracellular	507
Stirred tank	Batch, one-stage	2	5 ^b	Rootstock 41B/30	MeJA	Resveratrol 1.0 extracellular + 0.1 intracellular ϵ -viniferin and piceatannol extracellular	147
Stirred tank	Batch, one-stage	1.2	23	Gamay/20	DIMEB	Resveratrol 3.1 extracellular	506
Stirred tank			21		DIMEB + MeJA	Resveratrol 13.5 extracellular	506
Stirred tank	Fed-Batch, two-stages	10	7.25	V. labrusca Concord/30	β -CD + MeJA	Resveratrol 0.63 extracellular δ -viniferin 0.43 extracellular	51
Stirred tank	Fed-Batch, two-stage	5	23.8 ^c	V. labrusca Concord/30	MeJA	Resveratrol 0.28 extracellular ^c	509
Stirred tank	Fed-Batch, two-stages	5	17.7 ^c	V. labrusca Concord/30	MeJA	Resveratrol 1.26 extracellular ^c Pallidol 0.23 extracellular ^c δ -viniferin 0.08 extracellular ^c ϵ -viniferin 0.01 extracellular ^c	493
Stirred tank	Fed-Batch, two-stages	20	22.6 ^c	V. labrusca Concord/30	DIMEB+MeJA	Resveratrol 14.3 extracellular ^c Pallidol 0.6 extracellular ^c δ -viniferin 1.5 extracellular ^c	493
Bubble column V-shaped	Batch, one-stage	1.2	21	Gamay/20	DIMEB	Resveratrol 2.2 extracellular	506

Table 3 Resveratrol bioproduction by grapevine cell suspensions in bioreactors (continued)							
Bubble column V-shaped	Batch, one-stage	1.2	21		DIMEB +MeJA	Resveratrol 13.5 extracellular	506
Bubble column cylindrical	Fed batch three cycles, one-stage	5.8 x 3	25.7	Gamay/20	DIMEB +MeJA	Resveratrol 10.9 x 3 extracellular	506
Bubble column cylindrical	Fed batch three cycles, two-stages	5.8 x3	18.4	Gamay/20	DIMEB +MeJA	Resveratrol 11.1 x 3 extracellular	506

^aEstimated considering the dilution factor of flasks producing 460g/L biomass. ^bEstimated considering final biomass and fold increase. ^cEstimated considering that an inoculum of 15g DW/L⁴⁹³ is equivalent to an inoculum of 475g FW/L,⁵¹ thus conversion factor is DW=0.0315.FW. When no explicit data were stated in papers, these were calculated after the graphical material contained in them.*Estimated considering the dilution factor of flasks producing 460g/L biomass **Estimated considering final biomass (356g) increased by 3.56-fold