Phytoalexins from the Vitaceae: Biosynthesis, Phytoalexin Gene Expression in Transgenic Plants, Antifungal Activity, and Metabolism

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Resistance of plants to infection by phytopathogenic microorganisms is the result of multiple defense reactions comprising both constitutive and inducible barriers. In grapevine, the most frequently observed and best characterized defense mechanisms are the accumulation of phytoalexins and the synthesis of PR-proteins. Particular attention has been given here to stilbene phytoalexins produced by Vitaceae, specifically, their pathway of biosynthesis (including stilbene phytoalexin gene transfer experiments to other plants) and their biological activity together with fungal metabolism.

Keywords: Phytoalexins; resveratrol; Vitaceae; gene transfer; biological activity; metabolism

INTRODUCTION

Plants in their natural environment are challenged by large numbers of potentially pathogenic microorganisms, mainly fungi, bacteria, and viruses. The factors determining the resistance of plants against phytopathogens belong to a large arsenal of constitutive and inducible (active) defense mechanisms (*1*). The typical preformed, constitutive defenses are the structural barriers (waxes, cutin, suberin, lignin, phenolics, cellulose, callose, and cell-wall proteins) which are often rapidly reinforced upon the infection process. Active defense mechanisms mainly involve the oxidative burst, rapid and localized cell death (hypersensitive response), accumulation of phytoalexins, and synthesis of pathogenesis-related (PR) proteins. Defense response mechanisms are not only activated upon infection by pathogenic microorganisms, but can also be induced by abiotic stresses such as induction with UV-light, or by chemicals (respiratory inhibitors, surfactants, antibiotics, plant regulators, or the salts of heavy metals, as well as elicitors released by the pathogens or products resulting from the activity of fungal degrading-enzymes on host cell-walls) (*2*).

In grapevine, the most frequently observed and best-characterized defense reactions upon fungal infection are accumulation of phytoalexins and the synthesis of PR-proteins (*3*). Because grapevine is an agriculturally and economically important crop plant, the defense mechanisms of that plant against phytopathogenic microorganisms have attracted considerable attention. Among them is the phytoalexin production. Phytoalexins from the Vitaceae have been the subject of numerous studies during the past decade, because these compounds are thought to have implications in both phytopathology and human health. As a result, there is a huge increase in the literature dedicated to this particular class of phytoalexins. There have been 238 articles published on resveratrol in the last two years alone compared to 170 during the previous 10 years, with most of these papers concerning the role of resveratrol in human health.

Phytoalexins are low-molecular-weight antimicrobial secondary metabolites of wide interest (*2, 4, 5*). From a fundamental point of view, studies in phytoalexins provide a large field of investigation for biochemists and plant pathologists, especially concerning the aspects of biosynthesis of these compounds in plants and their metabolism by pathogenic microorganisms. Phytoalexins have been shown to possess biological activity against a wide range of pathogens and can be considered as markers for plant disease resistance. Although most phytoalexins are less phytotoxic than synthetic fungicides, they can accumulate in large quantities within plant tissues, far exceeding concentrations necessary to inhibit fungal growth (*6*).

The aspects developed here focus on the biosynthesis and the antifungal activity of grapevine phytoalexins together with their metabolism. Particular attention has also been given to stilbene phytoalexin gene transfer experiments to other plants.

PHYTOALEXINS FROM THE VITACEAE

Although phytoalexins display an enormous chemical diversity (for reviews see *2, 5, 7−9*) phytoalexins from the Vitaceae seem to constitute a rather restricted group of molecules belonging to the stilbene family (*10*), the skeleton of which is based on *trans*-resveratrol structure (3,5,4′-trihydroxystilbene) (**Figure 1**). In addition to resveratrol, other compounds con-

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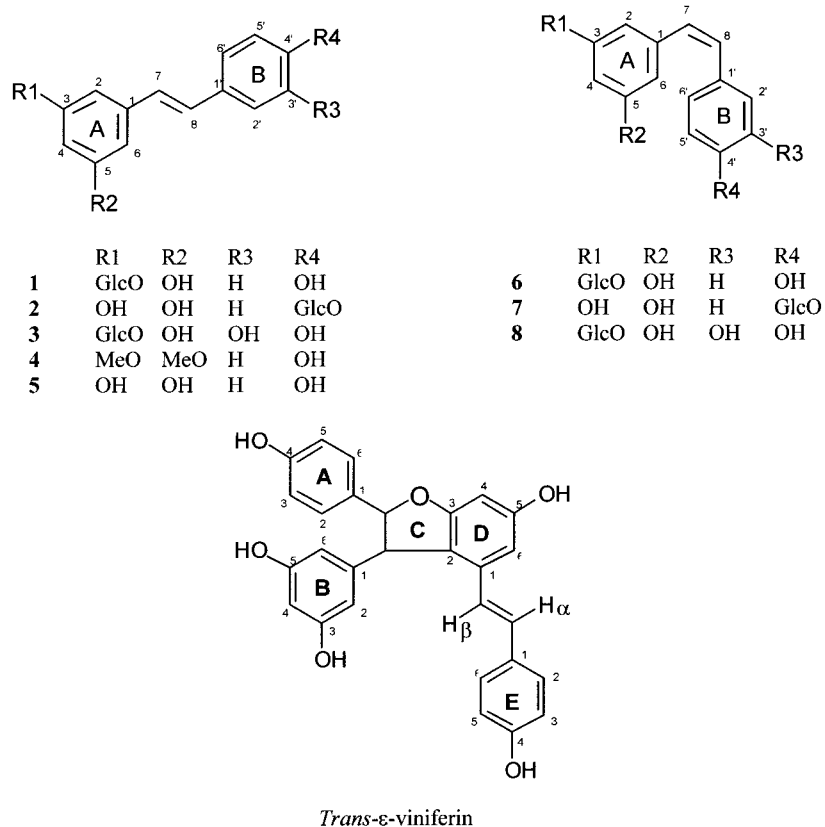‡ Université de Bourgogne.
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	R1	R2	R3	R4
1	GlcO	OH	H	OH
2	OH	OH	H	GlcO
3	GlcO	OH	OH	OH
4	MeO	MeO	H	OH
5	OH	OH	H	OH

	R1	R2	R3	R4
6	GlcO	OH	H	OH
7	OH	OH	H	GlcO
8	GlcO	OH	OH	OH

Trans-ε-viniferin

Figure 1. Chemical structures of stilbene phytoalexins. **1** and **6**, *trans*- and *cis*-piceid; **2** and **7**, *trans*- and *cis*-resveratroloside; **3** and **8**, *trans*- and *cis*-astringin, **4**, *trans*-pterostilbene; **5**, *trans*-resveratrol. Glc: glucosyl ($C_6H_{11}O_5$).

sidered as oligomers of resveratrol and termed viniferins have also been found in grapevine as a result of infection or stress. The major components of these appear to be ε-viniferin, a cyclic resveratrol dehydrodimer (*10*, *11*), and α-viniferin, a cyclic resveratrol dehydrotrimer (*12*). Simple stilbenes have been identified as well: *trans*-pterostilbene, a dimethylated resveratrol derivative (3,5-dimethoxy-4′-hydroxystilbene) (*13*, *14*), *trans*- and *cis*-piceid, a 3-*O*-β-D-glucoside of resveratrol (*15*, *16*), *trans*- and *cis*-astringin, a 3-*O*-β-D-glucoside of 3′-hydroxy-resveratrol and *trans*- and *cis*-resveratrol-oside, a 4′-*O*-β-D-glucoside of resveratrol (*17*) (**Figure 1**). Stilbenes are responsible for the bright blue fluorescence observed under long wavelength UV-light on grape leaf surfaces or grape berries following their accumulation within plant tissues (*18*, *19*).

BIOSYNTHESIS OF RESVERATROL AND STILBENE SYNTHASE GENE EXPRESSION

Stilbene phytoalexins, as flavonoid-type phytoalexins (*20*–*23*), are formed on the phenylalanine/polymalonate pathway (*10*), the last step of this biosynthesis pathway being catalyzed by stilbene synthase (**Figure 2**). Stilbene synthase (STS) (EC 2.3.1.95) produces the simple stilbene phytoalexins in one enzymatic reaction from coenzyme A-esters of cinnamic acid derivatives (*p*-coumaroyl-CoA in the case of resveratrol or dihydrocinnamoyl-CoA in the case of dihydropinosylvin, a stilbene not present in grapevine) and three malonyl-CoA units (*24*). STS was first purified from cell suspension cultures of *Arachis hypogea* (*25*). STS is encoded by a multigene family. Eight resveratrol-forming STS genes from grapevine (*pSV21*, *pSV25*, *pSV696*, and *pSV368* (*26*), and *Vst1*, *Vst2*, and *Vst3* (*27*)) and five pinosylvin-forming STS genes from pine (*PST-1*, *PST-2*, *PST-3*, *PST-4*, and *PST-5*) (*28*) are characterized. Three novel
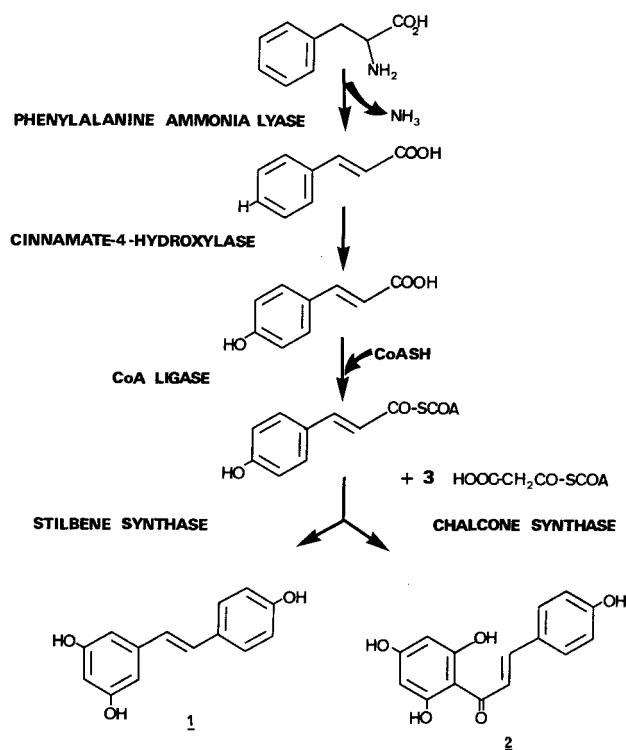

Figure 2. Biosynthetic pathway from phenylalanine to resveratrol, **1**, and chalcone, **2**.

STS genes (*pdsts1*, *pdsts2*, and *pdsts3*) have recently been isolated from the roots of *Pinus densiflora* (*29*), together with a new stilbene synthase gene from *Vitis riparia* cv Gloire de Montpellier (*30*). STS genes were grouped according to their

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responsiveness toward external signals including abiotic stresses or biotic signals originating from fungal cells (*28, 31*). STS cDNA and genomic clones have been described from Scots pine (*32*), groundnut (*33*), and grapevine (*34, 35*). Native STS is an homodimer of 90 kDa (with 43 kDa subunits) (*36*). STS is closely related to chalcone synthase (EC 2.3.1.74) (CHS), the key enzyme in flavonoid-type compound biosynthesis (*37*). STS and the consensus sequence of CHS from *Arachis hypogea* share a 70 to 75% identity at the protein level, the sequences differing by only 35 amino acid positions (*38*). Molecular analysis of cDNA and genomic clones of STS and their comparison with CHS suggests common evolutionary origin for these two enzymes. STS and CHS use the same substrates and catalyze the same condensing-type of enzyme reaction, but form two different products, respectively, chalcone, the first C15 intermediate in the C6C3C6 route, and simple stilbenes. The crystal structures of CHS (but not those of STS) have recently been obtained (*23, 39*). The structure of CHS complexed with resveratrol indicates how STS can use the same substrates and an alternate cyclization pathway to form resveratrol (*23*). Yamaguchi et al. (*40*) confirmed the close relationship between STS and CHS by describing cross reactions of CHS and STS overexpressed in *Escherichia coli* (i.e., resveratrol formation by CHS and, reciprocally, chalcone production by STS). STS and CHS contain, at the same position, a single essential cysteine residue (Cys 164), which most likely represents the active site (*41*). A single change of histidine residue (close to the active site) to glutamine is responsible for the substrate specificity in STS: in the STS that produce pinosylvin using cinnamoyl-CoA as a starter CoA ester, and in the STS that produce resveratrol using *p*-coumaroyl-coA, these positions are occupied by Gln-His and His-Gln, respectively (*42*).

Although a constitutive expression of STS was previously reported (*35*), expression of STS genes is often induced in response to biotic and abiotic stresses (see below). At a transcriptional level, it was shown that STS mRNAs accumulate in two waves: e.g., 6 and 20 h after treating cell suspensions of *V. vinifera* cv Optima with cell walls of *Phytophthora cambivora* (*27*). Similar results were previously obtained with grapevine cell suspensions elicited with cell walls of *B. cinerea* (*36*). These two peaks, differing in time, were thought to correspond to the expression of, at least, two different groups of STS genes: those expressed early but with a rapid degradation of the *m*RNAs produced and those expressed later and slowly activated, providing a more stable *m*RNA (*27*). Zinser et al. (*43*) observed that ozone-induced transient STS transcript levels reached their maximal values in two subsequent waves between day 1 and day 5 after ozone exposure. Adrian et al. (*44*) also described the occurrence of two peaks in the profile of STS mRNAs in *V. vinifera* in in-vitro-grown-leaves in response to induction with UV-light, the first one reaching a maximum 8 to 12 h after induction, while the second peak occurred 22 to 24 h after induction. Similarly, Douillet-Breuil et al. (*45*) noted the occurrence of two maxima in resveratrol accumulation in grapevine leaves treated by UV-light (i.e., 20 and 40 h, respectively, after induction).

Phytoalexin synthesis is induced in grapevine, as for many other phytoalexins (*46*), in response to a wide range of biotic and abiotic stress factors. Formation of the phytoalexin resveratrol was first described as a response to UV−C-irradiation in grapevine leaves and to various fungi as well (*Botrytis cinerea* Pers. Fr., *Plasmopara viticola*, and *Oidium tuckeri*, respectively the causal agents for gray mold, downy mildew, and powdery milew) (*13, 18, 19, 36, 44, 45, 47, 48*). It is now well-known that resveratrol can be synthesized by grapevine in response to other stresses: (i) induction by heavy metals such as aluminum or aluminum-containing fungicides (*49−54*), and (ii) induction by chemicals such as ozone (*43, 55, 56*). Interestingly, it has recently been shown that *V. vinifera* cells grown in vitro respond to methyljasmonate with enhancement of phytoalexin (piceid) accumulation, suggesting that jasmonate and its methylester may be key components of the signal transduction system involved in the formation of grapevine phytoalexins (*57*). Stilbene phytoalexins are produced by grape leaves, specifically at the abaxial leaf surface, and by grape berries, in the skin (*18, 19, 48*). Resveratrol synthesis steadily decreases in ripening grape berries (*19*) in relation to the decline in inducible STS gene expression (*58*), thus explaining the rise in susceptibility of mature fruits to *B. cinerea* infection (*59*).

STILBENE SYNTHASE (STS) GENE TRANSFER EXPERIMENTS

Great progress toward the design of crop plants with enhanced production traits, such as herbicide, insect, and disease resistance, is expected from the development of genetic transformation technology for plants. Promising results have already been obtained by introducing genes involved in microbial defense in plants such as, for example, PR protein genes in tobacco (*60, 61*), antibiotic genes in apple (*62, 63*), or phytoalexin genes (*64, 65*). Particularly, transformation of plants with STS genes has led to interesting applications in genetic engineering and has been studied intensively.

The first gene transfer experiments were performed with one complete STS gene from *Arachis hypogea* together with a chimeric kanamycin-resistant gene, introduced into tobacco (*Nicotiana tabacum*) via the transformation of protoplasts. Here, its expression resulted in resveratrol synthesis after induction with short-wavelength UV-light (*66*). Moreover, it was shown that the transfer of two grapevine STS genes (*Vst1* and *Vst2*) into tobacco confers that plant disease resistance (*67*), transformed regenerated tobacco plants indeed showing a higher resistance to *B. cinerea* infection than the wild type. This study constitutes the first report of a disease resistance resulting from foreign phytoalexin expression in a novel plant. Since this elegant and pioneering work, STS genes have been transferred to a number of plants including rice (*68*), tomato (*69*), alfalfa (*70*), kiwifruits (*71*), and barley and wheat (*72−74*), as well as grapevine (*75*).

In all experiments, the expression of single STS genes (isolated from grapevine or *A. hypogea*) was optimized by using heterologous promoters and enhancer elements to permit strong transcriptional activity. This was achieved by modifying STS genes with enhancer sequences from the Cauliflower mosaïc virus 35*S* (CaMV 35*S*) promoter (*66, 70−72*). STS genes were transferred to plants by means of *Agrobacterium* spp. in tomato (*69*), kiwifruits (*71*), and grapevine (*75*), or by particle bombardment in barley and wheat (*72−74*). Southern blot analyses of genomic DNA demonstrate that STS genes are stably integrated in the genome of transgenic plants. Expression of STS genes always leads to a significant increase in STS mRNA accumulation, STS activity, and resveratrol accumulation in transformed plants. Thomzik et al. (*69*) observed a maximum in the accumulation of STS mRNA 24 h after induction, and decreasing thereafter. These data significantly differ from those of Leckband and Lörz (*72*) who reported a maximum in the accumulation of STS mRNA 8 h after pathogen elicitation, followed by a decrease after 24 h, and a second maximum after 48 h: such a pattern in STS mRNA accumulation resembles
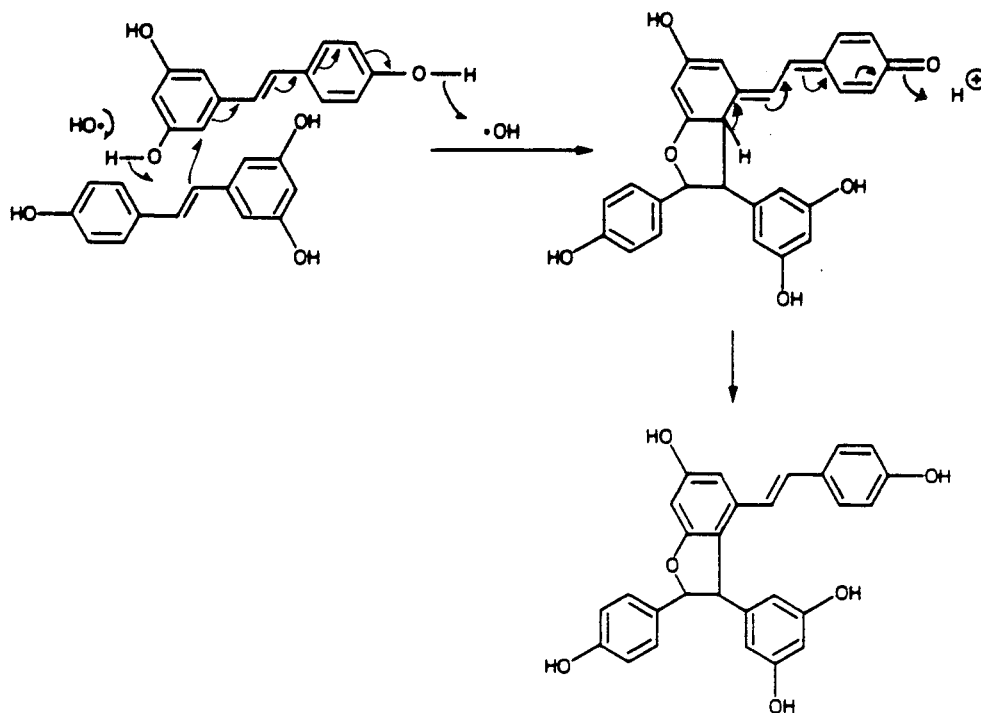
Figure 3. Mechanism of dimerization of resveratrol to ε-viniferin (redrawn with permission from ref *20*).

that described by Wiese et al. (*27*) (see above). By means of either HPLC methods (*71*, *73*, *75*), or ELISA assays using antisera raised against synthetic resveratrol (*66*, *67*, *69*), several studies showed a higher resveratrol accumulation in transgenic plants than in the wild type. Unexpectedly, the transfer of a grapevine STS gene under control of a heterologous inducible promoter (pathogen-inducible PR 10 promoter from alfalfa) back into grapevine 41B rootstock resulted in very high accumulation of resveratrol (5 to 100-fold the resveratrol levels found in nontransgenic control plants) associated with an increased resistance to the fungal pathogen *B. cinerea* (*75*). In contrast, Thomzik et al. (*69*) failed to demonstrate that STS gene expression results in resveratrol accumulation in rice plants, though these authors reported an increased resistance of transgenic plants to *Pyricularia orizae*.

Interestingly, besides the synthesis of a foreign phytoalexin, specifically resveratrol and glucosides, enhanced disease resistance against various pathogens has been obtained in transformed plants. Hain et al. (*67*) were the first to observe that STS gene expression resulted in enhanced disease resistance of tobacco to *B. cinerea*. Similar results reported increased resistance of transgenic plants to phytopathogenic microorganisms. Expression of STS gene enhanced resistance of rice to *Pyricularia orizae* (rice blast) (*68*), of tomato to *Phytophthora infestans* (*69*), of barley and wheat to *B. cinerea* (*72*), of wheat to *Oidium tuckeri* (powdery mildew) (*74*), and of alfalfa to *Phoma medicaginis* (*70*). The sole study reporting no increase in disease resistance in transgenic plants concerns the transformation of kiwifruits with a grapevine STS gene (*71*). Taken together these results imply a more general relevance of the STS system as a tool for engineering resistance to disease (*65*).

A very important question remains: which effects could a high resveratrol accumulation, resulting from STS gene expression, have on the physiology of transgenic plants? Though Hain et al. (*67*) reported that constitutive expression of STS gene under the control of the 35*S* promoter does not alter the normal growth of tobacco plants, Fischer et al. (*76*) observed that constitutive expression of STS gene (and resveratrol accumula-

tion) causes modification of the morphology and the color of the flowers and induces male sterility in tobacco. As many results have been obtained in vitro, it would also be important to determine whether transgenic plants can show disease resistance in the fields. For grapevine, for example, experiments are time-consuming, as it is necessary to wait three or four years for the first flowering period after planting. Such experiments with grapevine are now in progress (*75*).

BIOSYNTHESIS OF RESVERATROL DERIVATIVES

Oxidative dimerization of resveratrol units leads to several oligomers termed viniferins (*10*, *77*). Two of them are well characterized: ε-viniferin, a resveratrol dehydrodimer, and α-viniferin, a cyclic resveratrol dehydrotrimer (*12*, *77*). Although the occurrence of β-viniferin (a cyclic resveratrol tetramer) and γ-viniferin (an oligomer of high molecular weight) has already been suggested by Langcake and Pryce (*77*), these authors provided no direct evidence of that. It was shown that peroxidase is associated with *trans*-resveratrol oxidation to yield viniferins (**Figure 3**). Using horseradish peroxidase−hydrogen peroxide, Langcake and Pryce (*78*) demonstrated the formation of a grapevine phytoalexin mimic consisting of a resveratrol dehydrodimer analogous to ε-viniferin, but with a different oxidation coupling involving the hydroxyphenyl group situated in the 4′-position of the stilbene moiety. Oxidation of 4-hydroxystilbenes (including resveratrol) has been studied extensively (*79*−*81*). The most significant results show that resveratrol oxidation in grapevine is controlled by three peroxidase isoenzymes, i.e., A_1, B_3 (located in the cell wall and cell wall-free-spaces), and B_5 (located at the vacuolar level). These enzymes seem to be linked to both constitutive and inducible defenses of grapevine against fungi (*80*). Studies into the changes of stilbene concentration in grapevine leaves in response to UV−C elicitation indicated that the kinetics of production of resveratrol and ε-viniferin were very similar, synthesis of ε-viniferin rising according to resveratrol synthesis, but with a shift in the maximum peaks of synthesis of both compounds (i.e., 20 h and 40−50 h after induction, respectively) (*45*).

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Despite its high antifungal activity (*14*, *82*), biosynthesis of pterostilbene, the dimethylated resveratrol derivative, is not known. Specifically, there is, at this time, no direct evidence that pterostilbene derives from resveratrol and that methylation of the two hydroxyphenyl groups situated at the 3 and 5-position takes place on the stilbene skeleton. Our attempts to characterize a methyl-transferase leading in one step from resveratrol to pterostilbene, as is the case in the synthesis of pisatin from maackiain in pea (*83*), were unsuccessful.

ANTIFUNGAL ACTIVITY

Stilbenes are generally biologically active compounds that have antifungal activities against various pathogens: *Cladosporium cuccumerinum*, *Pyricularia oryzae* (*18*), *Plasmopara viticola* (*13, 84*), and *Sphaeropsis sapinea* (*85*). The antifungal activity of resveratrol has led to controversial studies, and the question of whether resveratrol is, or is not, a phytoalexin according to the definition of Müller and Börger (*86*), has been debated. The biological activity of resveratrol was first studied by Langcake and Pryce (*18*) using TLC assays. They established that the ED_{50} (i.e., effective dose or concentration required for 50% mortality) of resveratrol upon dormant conidia of *B. cinerea* or spores of *C. cuccumerinum* was more than 200 μg/mL and concluded that resveratrol, due to its low antifungal activity, should be considered as a precursor of compounds of higher fungitoxicity (viniferins and pterostilbene) rather than as a phytoalexin.

It now appears that Langcake and Pryce (*18*) used resveratrol concentrations far exceeding its solubility in their bioassays. Hoos and Blaich (*87*) reported inhibition of the radial growth of the mycelia of *B. cinerea* and *Phomopsis viticola* by resveratrol, but once again, these studies were performed using crystalline suspensions of high concentrations of this compound (ranging from 100 to 1000 μg/mL), leading to solubility problems such that accurate determination of the actual concentration of resveratrol in solution in the medium was impossible to obtain. Adrian et al. (*88*) established that resveratrol has real inhibitory effects on conidial germination of *B. cinerea* liquid cultures when used at concentrations ranging from 60 μg/mL (25% inhibition) to 160 μg/mL (100% inhibition), i.e., from 2.6 to 7 $\times$ 10^{-4} *M* (resveratrol solubility was ensured by addition of a minute quantity of ethanol; less than 4%). At a concentration of 60 μg/mL, resveratrol also reduced the germination of sporangia of *Plasmopara viticola* by 75% (*84*). These values correspond to the activity of other phytoalexins, which are generally active at concentrations of 10^{-4} to 10^{-5} M (for a review see *89* and *90*). The ED_{50} was 90 μg resveratrol/ mL (3.9 $\times$ 10^{-4} M) upon conidia of *B. cinerea*. Mycelial growth of *B. cinerea*, as determined by the hyphal mean length, decreased by 36% at 60 μg resveratrol/mL and by 82% at 120 μg/mL (*88*).

Pterostilbene, the dimethylated derivative of resveratrol, is 5-fold more active than resveratrol: it completely inhibits conidial germination at concentrations ranging from 52 to 60 μg/mL (ED_{50} = 18 to 20 μg/mL, i.e., 7 to 7.8 $\times$ 10^{-5} M) (*13*, *14*, *88*). The differences in the antifungal activities of two related stilbene compounds indicate that the in vivo methylation of hydroxyphenyl groups can potentially lead to increased biocidal activity in phenolics (*14*).

According to Langcake (*13*), ϵ-viniferin has an antifungal activity upon germination of *B. cinerea* conidia (ED_{50} = 36 μg/mL, 7.9 $\times$ 10^{-5} M) very similar to that of pterostilbene, which is the most toxic stilbene. ED_{50} values for resveratrol are comparable to the actvities of other phytoalexin-type

compounds: specifically, pisatin and glyceollin, two phytoalexins from the *Leguminosae,* have ED_{50} values of, respectively, 100 μg/mL (3.2 $\times$ 10^{-4} M) against *Phytophthora megasperma* (*91*) and 40 μg/mL (1.2 $\times$ 10^{-4} M) against *Aphanomyces euteiches* (*92*); scoparone, a phytoalexin from *Citrus* has an ED_{50} value for inhibition of *Phytophthora citrophthora* of 97 μg/mL (4.7 $\times$ 10^{-4} M) (*93*, *94*).

Stilbenes may also alter fungal morphogenesis. Treatment of conidia with sub-lethal or lethal concentrations of resveratrol (60$-$140 μg/mL) or pterostilbene (20$-$40 μg/mL) does indeed result in cytological abnormalities in *B. cinerea* conidia, including the formation of curved germ tubes, cessation of growth of some germ tubes with protoplasmic retraction in the dead hyphal tip cell, cytoplasmic granulation of conidia, disruption of the plasma membrane, or regrowth of a secondary or tertiary germ tube from the surviving conidium (*88*). At an ultrastructural level, Pezet and Pont (*14*) reported that pterostilbene added at a concentration of 128 μg/mL (5 $\times$ 10^{-4} M) to dormant conidia of *B. cinerea* induces strong modifications of the endocellular membrane system; specifically, it causes the rapid destruction of endoplasmic reticulum, and of nuclear and mitochondrial membranes, all these phenomena synchronously appearing with a complete cessation of respiration (i.e., 5 to 10 min after pterostilbene addition). Within 30 min, the cytoplasm is coagulated into numerous vacuoles and mitochondria are clear with a complete disorganization of the cristae. Destruction of the conidium ends (after 3 h) with the disruption of the plasma membrane.

Some of these features have previously been described for other phytoalexin/pathogen interactions (for reviews see *89* and *90*). For example, zoospores of *Phytophthora infestans*, *Phytophthora porriet*, and *Phytophthora cactorum* can develop cytoplasmic granulations, plasma membrane disruptions, and the leakage of cellular contents (*95*, *96*) in the presence of four terpenoid-type phytoalexins (rishitin, phytuberin, anhydro-β-rotunol, and solavetivone). These effects have also been observed in fungal cells treated with isoflavonoid-type phytoalexins (phaseollin and kievitone) (*97$-$100*). In addition, phaseollin and the phenanthrenes orchinol and dehydroorchinol have been reported to cause the death of apical fungal cells (*97*, *101*). Protoplasmic retraction in the hyphal tip cell of *B. cinerea* observed after exposure to resveratrol or pterostilbene can be explained by the fact that apical cells of hyphae are more susceptible to phytoalexins because of their weak wall, which facilitates the entry of these compounds into the cell. In comparison, the entry of phytoalexins into subapical and interstitial hyphal cells (which have mature walls) is generally more restricted (*98, 102$-$105*). Observations of regrowth from surviving conidia (production of a secondary or a tertiary germ tube) also suggest that cells can escape from the action of phytoalexins, with re-germination constituting a means of survival for conidia (*104*, *105*). Finally, one significant reaction to stress is the formation of curved germ tubes after resveratrol treatment. This asymmetric growth may correspond to the ability of stilbenes, namely resveratrol, to interact with tubulin, with resultant disruption of microtubule assembly at this level, as described by Woods et al. (*106*) working with various stilbenes based on combretastatin A-4 and used in cancer therapy. This mode of action is typical for many other fungicides, such as benomyl, a fungicide used for the control of gray mold in the vineyard (*107*).

The mode of action of hydroxystilbenes on fungal cells has been studied extensively by the group of Pezet (*14*, *82*, *108*). They suggested that 4'-hydroxystilbenes (especially those

presenting methoxy-groups or electron-attracting substituents such as chlorine at the 3-, 3,4-, or 3,5-positions of the stilbene ring) play an important role in the formation of charge transfer complexes, favoring contact and affinity with (membrane) proteins and acting as uncoupling agents of electron transport and photophosphorylation. At a subcellular level, it has recently been reported that hydroxystilbenes, such as resveratrol and piceatannol, are capable of inhibiting some fungal ATPases and inducing the dissociation of chaperones and co-chaperones, two proteins frequently associated with the cytoskeleton (*109*).

The fact that stilbenes have a substantial antifungal activity suggests that these compounds can intervene in the resistance against fungal diseases affecting grapevine and be used as indicators of resistance to disease. Pool et al. (*110*) at Cornell University were the first to seek to establish a relationship between phytoalexin production potential and resistance to cryptogamic diseases in grapevine. This study has shown that both the speed and the intensity of resveratrol synthesis are positively correlated with the resistance of grapevine varieties to fungal diseases, namely to infection by *B. cinerea* or *Plasmopara viticola*. Unfortunately, the production of resveratrol by grapevine growing in the field has proved to be very sensitive to a wide range of environmental factors, thus limiting its use as a marker for disease resistance (*111*). The most optimistic results were obtained by our group concerning the development of a method using resveratrol assessment as a selection criterion to screen in vitro-grown grapevine for resistance to gray mold (*112*). Eleven of the thirteen tested grape genotypes showed a good correlation ($R = 0.831$) between resveratrol production (as induced by UV−C elicitation) and gray mold resistance. In many instances, a close relationship between phytoalexin accumulation and resistance to diseases has also been demonstrated (*5*, *9*, *90*, *113*).

FUNGAL METABOLISM

If stilbene-type phytoalexins represent a contributory factor in the resistance of grapevine to fungal attacks, the capacity of the pathogen to metabolize antifungal compounds released by the host could also play a significant role in the outcome of the interaction between grapevine and fungal pathogens. According to Mansfield (*90*), the net accumulation of phytoalexins within plant tissues infected by various pathogens is probably controlled by a balance which may result, on one hand, from the ability of the host cells to resist colonization by creating an inhibitory barrier to the parasite and, on the other hand, from tolerance of the pathogen to antifungal compounds produced by the plant and from its ability to metabolize (or detoxify?) the phytoalexins to which it is exposed. Thus, the resistance of plants to infection depends, in part, on the phytoalexin production/degradation balance following attack by the pathogen. A variety of factors can alter this balance in favor of either the plant or the host. Some studies have demonstrated the importance of stilbene metabolism for the pathogenicity of fungi on grapevine. Works in this area have focused only on the interaction between grapevine and *B. cinerea*. Data concerning stilbene metabolism by other grape pathogens are still scarce (*84*).

Hoos and Blaich (*87*) were the first to report the oxidative degradation of resveratrol by a fungal enzymatic activity (attributed to a laccase) present in *B. cinerea* and secreted in the culture medium of this fungus. This laccase-like stilbene oxidase was purified (*114*). Stilbene oxidase (STOx) presents two major isoforms with p*I* 4.35 and 4.3 and a molecular mass of 32 kDa. STOx can be inhibited by some phenolics of grape berries (*115*). Localization of this enzyme in the fungal cell

was demonstrated by Adrian et al. (*116*) using a cytochemical reaction with syringaldazine as a substrate for laccase. Syringaldazine produces unique electron-opaque deposits after oxidation that are visible by transmission electron microscopy in the cytoplasm and also in the cell wall and surrounding mucilages (which is expected because laccase is an extracellular enzyme), but never at the vacuolar level. It was deduced from these observations that the laccase-mediated oxidation of stilbenes takes place in the cytoplasm.

Pezet (*114*) suggested that STOx catalyzes the oxidation of resveratrol to ε-viniferin, based on chromatographic data. Nevertheless, further studies carried out first by our group have shown that resveratrol metabolism by STOx of *B. cinerea* (strain SP 1) includes an oxidative dimerization process (involving the 4′-hydroxyphenyl group of one stilbene unit) leading to a resveratrol dehydrodimer (obtained in 60% yield), slightly different from ε-viniferin (*117*). ^{1}H NMR and ^{13}C NMR showed that this metabolite is a dehydrodimer of resveratrol with both a dihydrobenzofuran and pentaphenolic structure as indicated in **Figure 4**. Similar data were obtained with the dimethylated resveratrol derivative, pterostilbene (*118*). These results were confirmed by Cichewicz et al. (*119*), who showed that, in addition to the dehydrodimer, metabolism of resveratrol by *B. cinerea* (strain ATCC 11 542) can lead to other oxidized resveratrol dimers, such as restrytisols (A to C), leachinol F, and pallidol.

Simple stilbenes thus undergo oxidative dimerization during their metabolism by *B. cinerea* generally involving the hydroxyphenyl group situated at the 4′ position of the stilbene skeleton. Dimerization of stilbene monomers may include as an intermediate compound a quinone that leads to a radical cation with a substituted *p*-hydroxyphenyl group, thus explaining chemical reactivity of stilbene phytoalexins (*120*). The 4′-hydroxyphenyl group is important for the metabolism of simple stilbenes, confirming the findings of Pezet (*114*) according to which a free or a hydroxylated 4′-position of the stilbenic ring is required for enzymatic oxidation of stilbenes. Grapevine phytoalexin metabolism by *B. cinerea* appears to be quite different from the reaction mechanisms already described for phytoalexin metabolism by fungi, which mainly include reactions of monoxygenation, demethylation, reduction, and hydration (for a review see *121*). Still at question are whether the degradation of stilbene monomers by laccase of *B. cinerea* corresponds to a detoxification process, and which role the oxidative degradation of grapevine phytoalexins plays in the *B. cinerea*−grapevine interaction. In the case of resveratrol or pterostilbene, dimerization by a laccase-like STOx of *B. cinerea* results in stilbenes of high molecular weights (twice that of the monomeric form), which are barely soluble in water, preventing evaluation of their biological activity (*117*, *118*). Thus, metabolism of phytoalexins by *B. cinerea* may result in insoluble products allowing the fungus to escape from the action of grapevine phytoalexins. This is not a common feature in phytoalexin metabolism, because fungal conversion of phytoalexins usually, though not always, yields products increasing in water solubility (*121, 122*). Insolubilization of stilbene oligomers resulting from STOx activity is likely, as Adrian et al. (*116*) have observed a brown coloration at the vacuolar level in *B. cinerea* conidia, probably corresponding to the accumulation of resveratrol oxidation products. This was confirmed by the characterization, at the ultrastructural level, of spherical vesicles in the vacuole or along the vacuolar side of the tonoplast. These intriguing vesicles, whose size was not uniform (varying from 0.1 to 0.4 μm), were either well-delimited (by an electron-dense ring different from
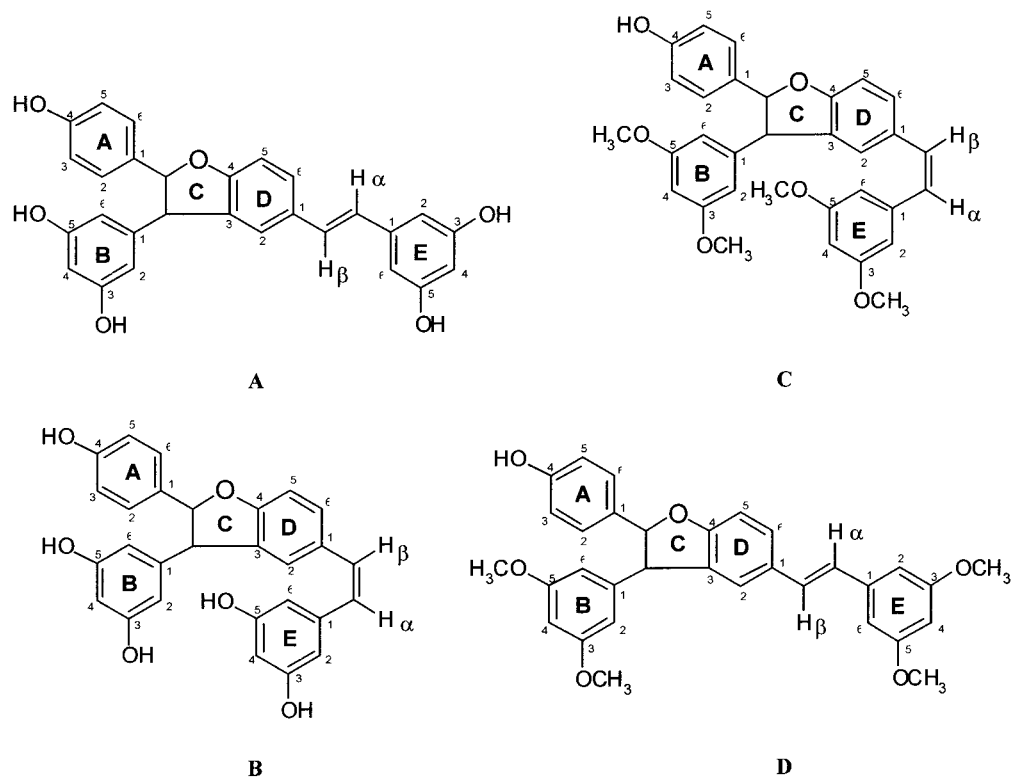
Figure 4. Chemical structures of the stilbene dehydrodimers formed by *B. cinerea.* **A**, trans- and **B**, cis-isomers of the resveratrol dehydrodimer. **C**, cis- and **D**, trans-isomers of the pterostilbene dehydrodimer. Only the trans forms of both dehydrodimers are naturally occuring metabolites produced by laccase-like stilbene oxidase of *B. cinerea.* Cis-dehydrodimers were obtained by photochemical isomerization after irradiation under long wavelength UV light of the trans forms ((**A** and **B** reproduced with permission from ref. *117* (copyright 1998, Elsevier Science); **C** and **D** reproduced with permission from ref. *118* (copyright 1999, American Phytopathological Society)).

a membrane), fully or partially filled with a coarsely granular material, or even empty (*116*). It was proposed that these vesicles correspond to the accumulation of products linked to the laccase-mediated oxidation of resveratrol, which occurs in the cytoplasm of the conidium; the resulting compounds then enter the vacuole where they accumulate.

Of prime concern for phytopathologists is the need to know whether the ability of a pathogen to metabolize phytoalexins relates to its pathogenic potential on that plant. Specifically, Sbaghi et al. (*123*) found that there are marked differences in the ability of isolates of *B. cinerea* to metabolize stilbene phytoalexins (i.e., resveratrol and pterostilbene): isolates with a high capacity to degrade phytoalexins, those with a limited capacity for degradation, and those totally unable to do so. It was interesting to observe that all strains of *B. cinerea* which can degrade stilbene phytoalexins were highly or moderately pathogenic on grapevine, whereas those that were unable to metabolize phytoalexins appeared to be nonpathogenic under these conditions. These experiments are in good agreement with those previously published concerning the relationship between pathogenicity and phytoalexin metabolism (see for example (*124*) in the case of phaseollin; (*125*) in the case of rishitin; (*126*) for the phytoalexin kievitone; and (*127*) for the phytoalexins maackian and medicarpin), thus showing the importance of phytoalexin detoxification for fungal pathogenicity (*122*).

CONCLUSIONS

Phytoalexins have long been recognized as being important in the defense mechanisms of plants against phytopathogenic microorganisms. Their potential biological properties have stimulated a ferment of activity concerning the biosynthesis and the metabolism of these compounds in the plant. Research has gone forward because these compounds are thought to help agriculturally and economically important crop plants withstand colonization by pathogens. In many instances, a close correlation has been found between phytoalexin production and resistance to diseases. And what of grapevine phytoalexins? There is no doubt that resveratrol plays an important role in the resistance of grapevine to colonization by fungi, and our understanding of the genes (and their regulation) involved in stilbene biosynthesis is rapidly expanding.

The past several years have also witnessed intense research devoted to the role of stilbenes (which are also present in wines) and, among them, resveratrol, in human health because of their protective effects against cardiac ailments and cancer. The potential therapeutic value of resveratrol has stimulated research activities on the occurrence of this molecule in grapes and wines. Because of its capacity to confer disease resistance in grapevine, as well as its outstanding biological properties, most interest has now centered upon STS gene transfer from grapevine to numerous plants with the objectives of increasing their tolerance to pathogenic microorganisms and improving the nutritional quality of food products through the expression of pharmaceutically active compounds in plants incapable of synthesizing resveratrol. Some optimistic data have been obtained in this area, showing that disease resistance does indeed result from foreign phytoalexin expression in a novel plant.

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