

Beta-aminobutyric acid-induced resistance in grapevine against downy mildew: involvement of pterostilbene

Ana R. Slaughter · Mollah Md. Hamiduzzaman · Katia Gindro · Jean-Marc Neuhaus · Brigitte Mauch-Mani

Abstract BABA, a non-protein amino acid, was used to induce resistance in grapevine against downy mildew. BABA-induced resistance was observed in the susceptible cv. Chasselas as well as in the resistant cv. Solaris. Following BABA treatment, sporulation of *Plasmopara viticola* was strongly reduced and the accumulation of stilbenes increased with time following infection. Induction of *trans*-piceide, *trans*-resveratrol and, more importantly, of *trans*- ϵ - and *trans*- δ -viniferin and *trans*-pterostilbene was observed in BABA-primed Chasselas. On the other hand, induction of *trans*-resveratrol, *trans* δ -viniferin and *trans*-pterostilbene was observed in BABA-primed Solaris. The accumu-

lation of stilbenes in BABA-primed Solaris was much higher than that found in BABA-primed Chasselas. Furthermore, BABA-treatment of Solaris led to a rapid increase in transcript levels of three genes involved in the phenylpropanoid pathway: phenylalanine ammonia lyase, cinnamate-4-hydroxylase and stilbene synthase. BABA-primed Chasselas showed increased transcript levels for cinnamate-4-hydroxylase and stilbene synthase. Here we show that pre-treatment of a susceptible grapevine cultivar with BABA prior to infection with *P. viticola* primed the accumulation of specific phytoalexins that are undetectable in non-BABA-primed plants. As a result, the susceptible cultivar became more resistant to downy mildew.

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Abbreviations

BABA beta aminobutyric acid
BABA-IR BABA-induced resistance

Introduction

Downy mildew caused by *Plasmopara viticola* is one of the most serious diseases in vineyards worldwide. Both susceptible and resistant cultivars can be colonised by *P. viticola* zoospores, but in resistant ones, the development of the parasite is rapidly

inhibited. The most cultivated grape cultivars (*Vitis vinifera*) are susceptible to *P. viticola* and the control of downy mildew requires regular fungicide applications. The application of copper-containing fungicides to control downy mildew causes accumulation of this heavy metal in soil and groundwater resulting in toxic effects to the environment. Unfortunately, the replacement of such copper-based fungicides by synthetic fungicides with specific modes of action has promoted the development of resistant isolates of *P. viticola* (Matasci et al. 2008). For these reasons, alternative strategies are needed.

In grapevine, the most frequently observed and best-characterised defence reactions upon fungal infection are the deposition of lignin (Dai et al. 1995) and other phenolic compounds, increased peroxidase activity, accumulation of stilbene phytoalexins and the synthesis of pathogenesis-related (PR) proteins (Derckel et al. 1999). These defence mechanisms also seem to be present in susceptible cultivars, but in general, they are not activated or are delayed during the infection process. Because grapevine is an agriculturally and economically important crop plant, the defence mechanisms of that plant against pathogenic microorganisms, including phytoalexin production, have attracted considerable attention. Phytoalexins from the *Vitaceae* have been the subject of numerous studies, because these compounds are thought to have implications in both phytopathology and human health (Jeandet et al. 2002).

Phytoalexins are low molecular weight antimicrobial secondary metabolites (Kuc 1995). They have been shown to possess biological activity against a wide range of pathogens and can be considered as markers for plant disease resistance. Although phytoalexins display an enormous chemical diversity, phytoalexins from the *Vitaceae* seem to constitute a restricted group of molecules belonging to the stilbene family, the skeleton of which is based on the *trans*-resveratrol structure (3,5,4'-trihydroxystilbene) (Fig. 1a) (Jeandet et al. 2002). Other compounds considered as oligomers of resveratrol and termed viniferins have also been found in grapevine as a result of infection or other stresses. Resveratrol is also glycosylated as piceide (5,4'-dihydroxystilbene-3-*O*- β -glucopyranoside) (Fig. 1b) and pterostilbene (3,5-dimethoxy-4'-hydroxystilbene) (Fig. 1c) is a dimethylated resveratrol derivative (Jeandet et al. 2002). Recently, it was shown that an isomer of ϵ -viniferin (Fig. 1d), δ -viniferin (Fig. 1e), is one of the

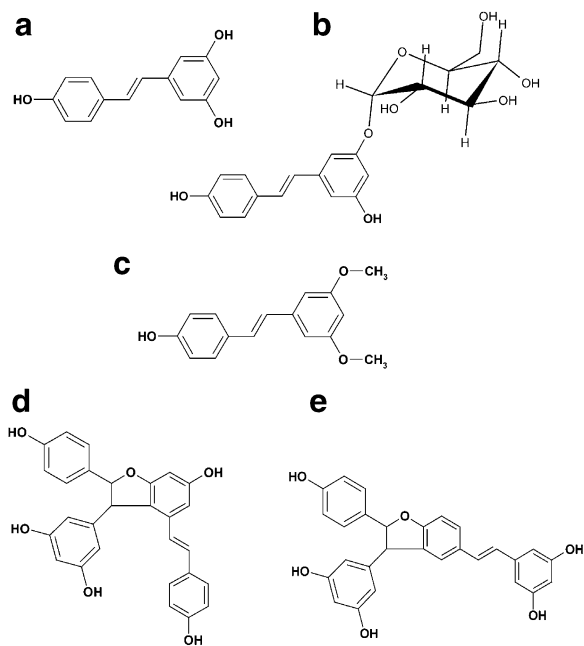


Fig. 1 Chemical structures of stilbene phytoalexins: **a** resveratrol, **b** piceide, **c** pterostilbene, **d** ϵ -viniferin and **e** δ -viniferin

major stilbenes produced from resveratrol oxidation in grapevine leaves infected by *P. viticola* (Pezet et al. 2003). Pezet et al. (2004b) tested the toxicity of these stilbenes against zoospores of *P. viticola* and found that δ -viniferin and pterostilbene were the most toxic stilbenes.

The phenylpropanoid pathway is an important pathway in secondary plant metabolism, yielding a variety of phenolics with structural and defence-related functions. These phenolic compounds include lignins, phenolic acids, flavonoids and stilbenes. In addition, enzymes such as phenylalanine ammonia lyase (PAL; EC 4.3.1.5), cinnamate-4-hydroxylase (C4H; EC 1.14.13.11) and 4-coumarate:coenzyme A ligase (4CL, EC 6.2.1.12) are considered to be crucial to phenylpropanoid metabolism. A number of reports have shown that phenylpropanoid derivatives are capable of protecting plants against various biotic (infection by viruses, bacteria, fungi) and abiotic (low and high temperatures, UV-B light, wounding) stresses (Sgarbi et al. 2003; Solecka and Kacperska 2003). Stilbene synthase (STS) (EC 2.3.1.95) catalyses the last step of the phenylpropanoid biosynthesis pathway leading to the formation of stilbene phytoalexins. Expression of STS genes is often induced in response to biotic and abiotic stresses (Jeandet et al. 2002).

In recent years, much attention has been focused on the activation of a plant's own defence system, known as induced resistance (Sticher et al. 1997). The non-protein amino acid β -aminobutyric acid has previously been shown to induce resistance against many different oomycetes and to be effective in inducing resistance against various downy mildews (reviewed in Jakab et al 2001; Cohen 2002). It has been speculated that BABA deteriorates penetrated host cells, blocking the translocation of nutrients into the haustoria, therefore prohibiting further mycelial growth and sporangial production (Steiner and Schönbeck 1997). Experiments with ^{14}C -labelled BABA showed that it was not metabolised in tomato (Cohen and Gisi 1994) or in *Arabidopsis* (Jakab et al. 2001), ruling out the involvement of a BABA metabolite acting as an antimicrobial compound in the plant. BABA-mediated resistance is therefore most likely to be based on the activation of host resistance mechanisms. Recently, in grapevine, it has been shown that callose deposition as well as defence mechanisms depending on the phenylpropanoid and the jasmonic acid (JA) pathways all contributed to BABA-IR (Hamiduzzaman et al. 2005).

In order to obtain a clearer picture of BABA-IR in grapevine against downy mildew, we looked at the involvement of stilbene phytoalexins and the expression of genes encoding enzymes involved in the phenylpropanoid pathway. Different staining techniques were used to visualise the biochemical changes that occurred at the cellular level between BABA and water-treated resistant and susceptible cultivars. After treatment of plants with BABA and water, we performed quantitative HPLC analysis of stilbenes and investigated the level of expression of the genes involved in the biosynthesis of stilbenes (PAL, C4H and STS) by quantitative real-time PCR.

Materials and methods

Plant material

Cuttings of *V. vinifera* cvs Chasselas and Solaris, the latter resulting from a cross of Merzling x GM 6493, carried out by the Weinbauinstitut in Freiburg, Germany, are sensitive and resistant to *P. viticola*, respectively. They were obtained from the grape collection at the Swiss Federal Research Station for

Plant Production in Changins, Switzerland and were grown in glasshouses. The cuttings were maintained in growth chambers (16 h light at 22°C, 8 h dark at 18°C and 60% relative humidity) until they had five fully developed leaves. They were then used for subsequent treatments and artificial inoculations.

Treatment and inoculation of plants

Cuttings from both cultivars were either soil drenched with an aqueous solution of BABA (1 mM) or with water (Hamiduzzaman et al. 2005) 2 days prior to inoculation with *P. viticola*. For the inoculum, leaves infected with *P. viticola* were harvested and sporangia were collected by vacuum aspiration, as described by Gindro et al. (2003). The abaxial leaf surfaces were inoculated by spraying an aqueous suspension of sporangia (5×10^4 sporangia ml^{-1}). The plants were then covered with transparent plastic bags and placed in growth chambers under the conditions described above. Samples were collected from both BABA- and water-treated leaves 0, 3, 7, 24, 48 and 72 h after inoculation and either used immediately for further analysis or frozen in liquid nitrogen and stored at -80°C . The experiments were carried out in duplicate.

Microscopic examination

Mycelia, sporangiophores and sporangia in inoculated leaves were stained with lactophenol-trypan blue (Keogh et al. 1980) and examined with bright field microscopy. Leaf material was placed on a glass slide in water, covered with a coverslip and examined with an epifluorescence microscope with an UV excitation filter (BP 340–380 nm, LP 425 nm). Blue fluorescence was used as an indicator of the presence of resveratrol (Dai et al. 1995). Flavonoid accumulation was visualized by using Wilson's reagent (Dai et al. 1995); leaves were immersed for 15 min, mounted in glycerol (75%) and examined under UV light with an epifluorescence microscope (BP 340–380 nm, LP 425 nm). Flavonoids fluoresced yellow and gallic acid derivatives (GAD) fluoresced blue.

Preparation of samples and HPLC analysis

At 0, 3, 7, 24, 48 and 72 h post-infection (hpi), three pieces of leaves were excised from inoculated leaves. Three replicates were made for each time point, each

cultivar and each treatment. Leaf samples were weighed and placed in a microfuge tube and 50 μ l of MeOH were added. The tubes were placed in a thermo-regulated shaker at 60°C for 10 min and then placed on ice for 5 min. The methanolic extracts (30 μ l) were analysed for stilbenes as described by Pezet et al. (2003).

Preparation of cDNAs

Total RNA was isolated from frozen leaf tissue using a modified CTAB extraction and lithium chloride precipitation method according to Iandolo et al. (2004). The quantity of total RNA was determined with a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA); in addition the quality of RNA was verified by the absorbance ratios (A260/A280) of 1.8 to 2.0. For quantitative real-time PCR analysis, RNA was treated with Turbo-DNase I (Ambion) according to the manufacturer's instructions. For cDNA synthesis, 1 μ g of RNA was reverse-transcribed using oligo(dT)₁₈ and Superscript III reverse transcriptase (Invitrogen Life Technologies) following the instructions of the manufacturer.

Real-time PCR for expression analysis of *CAH*, *PAL* and *STS*

Expression analysis of the genes *VvCAH*, *VvPAL* and *VvSTS* was done by real-time PCR, using the SYBER green method on an iCycler (Bio-Rad) real-time cycler. Each PCR reaction (20 μ l) contained 0.25 mM of each primer, cDNA and 1x Absolute QPCR SYBR Green mix (ABgene). The thermal cycling conditions were 95°C for 15 min followed by 95°C for 30 s, 56°C for 30 s, and 72°C for 35 s for 40 cycles, followed by a melt cycle from 60°C to 95°C. The primers used were as follows: *VvCAH*-F (5'-AGTCCAAGTCACCGAGCCTGAT-3') and *VvCAH*-R (5'-TAGCAAGCCACCATGCGTTCAC-3') for *VvCAH* (gene fragment obtained from a suppressive subtractive hybridisation SSH library constructed from *P. viticola*-infected grapevine), *VvPAL*-F (5'-TTGGTGCCACTTCACATAGGAG-3') and *VvPAL*-R (5'-AATCTGATGCCGGAGTAGCCTT-3') for *VvPAL*, and *VvStSy*-F (5'-CTCGAACCATCCGTCA GAAGAG-3') and *VvStSy*-R (5'-CCTACGATTA

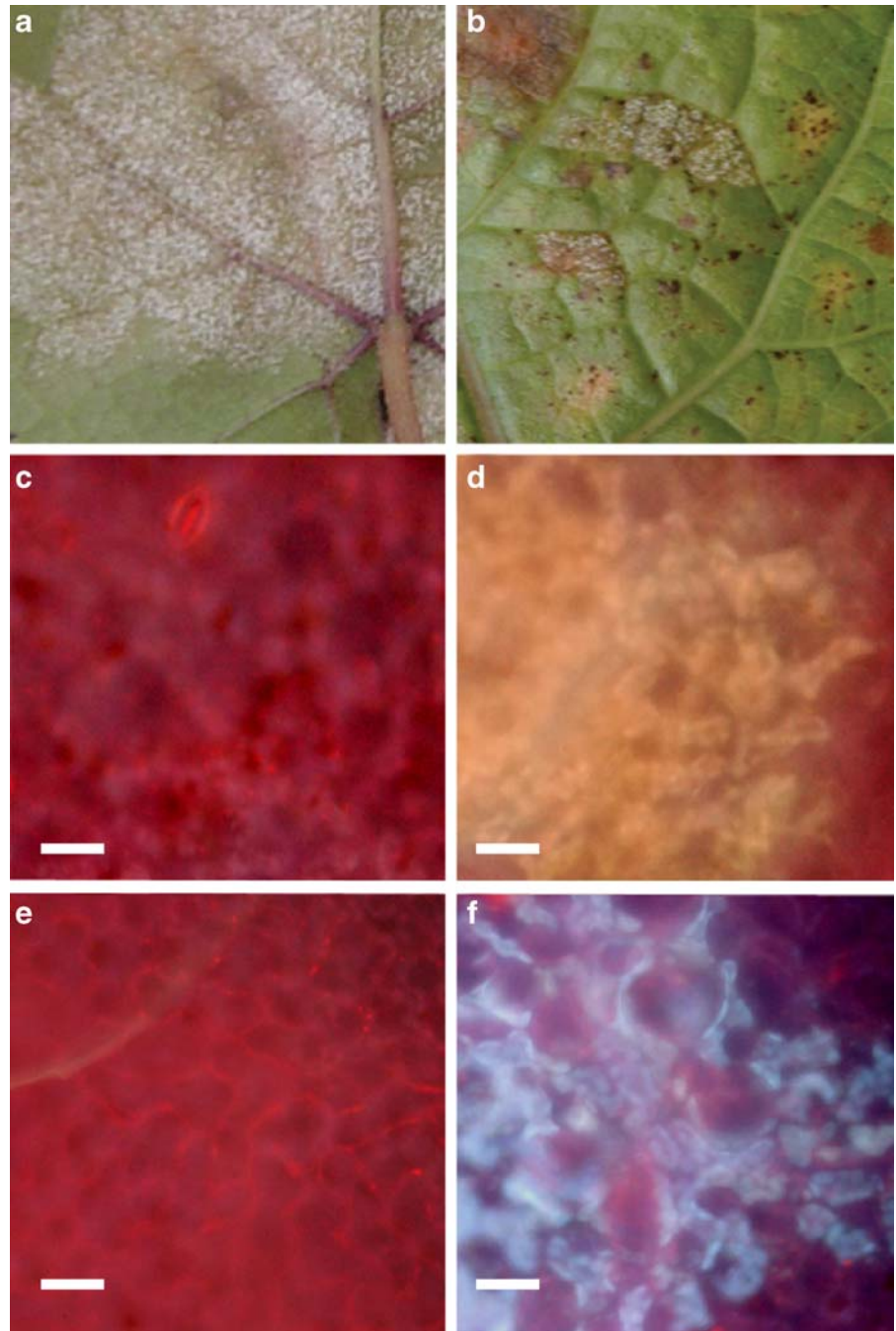
CAGCTGCAGACC-3') for *VvSTS*. With all cDNAs used, the above primer sets gave single PCR products, which were verified by determining the melt curves for the products at the end of each run and by analysis of the products using gel electrophoresis. The efficiency of the primers was tested in preliminary experiments with serial dilutions of cDNA samples and maintained an E value of between 0.97 and 0.98. The expression of the three genes was normalised relative to Elongation Factor 1- α (*VvEF1- α*) using the primers *VvEF1*-F (5'-GAACGTTGCTGTGAAG GATCTC-3') and *VvEF1*-R (5'-CGCCTGTCAACCT TGGTCATGA-3'). All samples were measured in triplicate, every run included the *VvEF1- α* control for each sample, and experiments were repeated twice. The Gene-X software (Bio-Rad) was used to calculate the mean normalised expression of the genes (Vandesompele et al. 2002).

Results

Microscopic observation of the biochemical changes in infected grapevine cuttings treated with or without BABA

Successful infection with *P. viticola* in susceptible Chasselas becomes apparent about 6 days after infection as a white, downy growth, mostly on the lower side of the leaves where *P. viticola* emerged from the stomata. Sporulation of *P. viticola* was strongly reduced in BABA-primed cuttings (Fig. 2b) compared to water-treated control plants (Fig. 2a) and the infection sites on BABA-primed cuttings were surrounded by necrotic groups of cells (Fig. 2b). Extensive hyphal growth and spread were observed within 3 days inside the leaf tissue of water-treated cuttings, whereas hyphal growth and spread of *P. viticola* was reduced in BABA-primed cuttings (data not shown). The biochemical changes at the cellular level were analysed at different time points (1–5 dpi) in BABA- and water-treated plants by using different staining techniques. Infected, non-BABA-primed plants displayed only the red fluorescence of chlorophyll in both plants stained with Wilson's Reagent (Fig. 2c) or unstained plants (Fig. 2e) when visualised under UV light. However, infected, BABA-

Fig. 2 Development of *P. viticola* and accumulation of stilbene compounds in grapevine (Chasselas). Plants were soil drenched with water (**a, c, e**) or 1 mM BABA (**b, d, f**) 2 days prior to challenge inoculation with sporangia of *P. viticola* (5×10^4 sporangia ml^{-1}). **a** White sporulation of *P. viticola* on water-treated cutting (6 dpi); **b** development of necrosis surrounding infection sites in BABA-primed cutting (6 dpi). Infected leaves were stained with Wilson's reagent and analysed by epifluorescence microscopy (**c** and **d**) or analysed by autofluorescence without prior staining (**e** and **f**). Water-treated controls show red chlorophyll fluorescence (**c** and **e**). Yellow fluorescence indicates flavonoid accumulation (**d**) and blue auto-fluorescence indicates presence of resveratrol in the tissues. Bars=50 μm



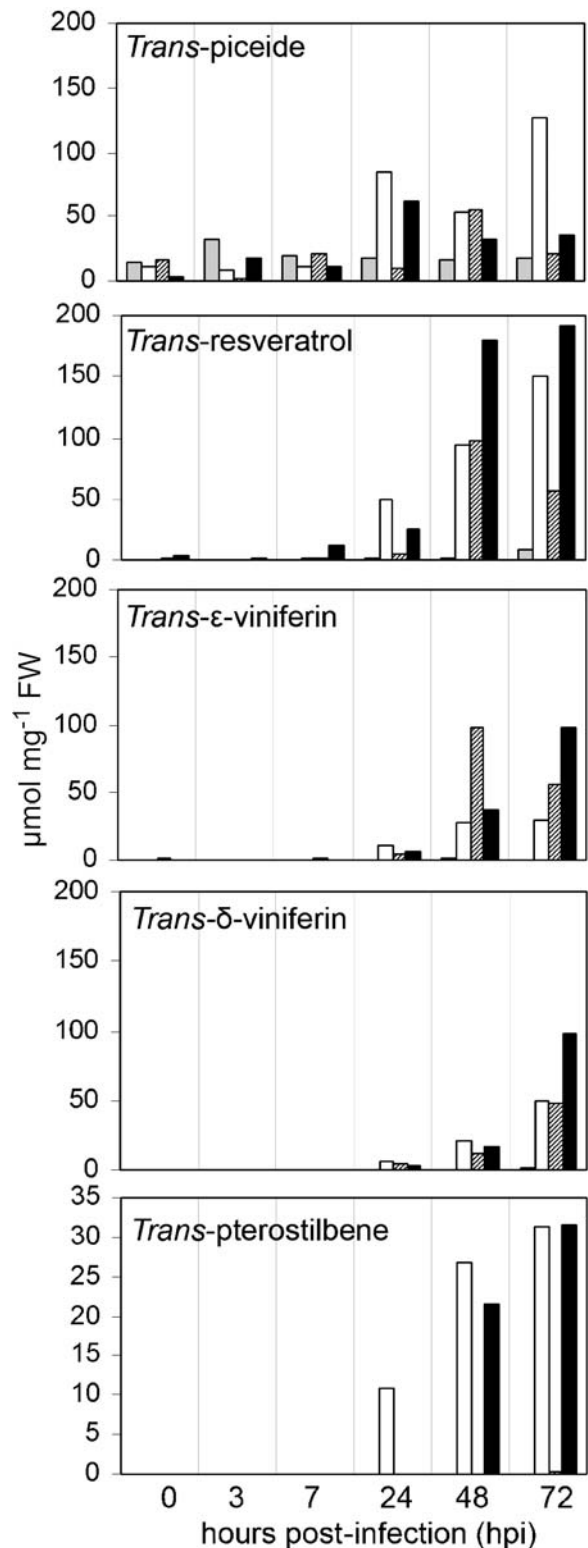
primed plants displayed a yellow fluorescence when stained with Wilson's Reagent (Fig. 2d) indicating the possible presence of flavonoids. The blue autofluorescence of infected, BABA-primed plants on the other hand suggests an accumulation of resveratrol in the tissues (Fig. 2f).

Accumulation of stilbene phytoalexins in *P. viticola* infected grapevine cuttings treated with and without BABA

The *trans*-form of five major stilbene phytoalexins: ϵ -viniferin, δ -viniferin, piceide, resveratrol and pter-

ostilbene were quantified. *Trans*-piceide was the only phytoalexin detected in both BABA- and water-treated Chasselas and Solaris for the six investigated time points (Fig. 3a). There was a significant increase in *trans*-piceide in BABA-primed Chasselas cuttings compared to the water-treated cuttings with the highest concentration occurring at 72 h pi. In contrast, *trans*-piceide increased significantly in BABA-primed Solaris cuttings compared to water-treated cuttings with the highest concentration occurring at 24 h pi. With the BABA concentrations used for our experiments no direct induction of phytoalexins was observed and accumulation occurred only in infected or primed/infected plants. During the 72 h analysis, *trans*-resveratrol was quantitatively the most abundant stilbene produced in BABA-primed Chasselas and Solaris (Fig. 3b). In both BABA-primed Chasselas and Solaris there was a marked increase in *trans*-resveratrol starting at 24 h pi and accumulating quantitatively to about 150 and 191 $\mu\text{mol mg FW}^{-1}$ for Chasselas and Solaris, respectively, at 72 h pi. Very slight amounts of *trans*-resveratrol were detected in water-treated Chasselas at 72 h pi ($8 \mu\text{mol mg FW}^{-1}$), whereas in water-treated Solaris, *trans*-resveratrol was detected at 24 h pi with a significant increase at 48 h (5 and 97 $\mu\text{mol mg FW}^{-1}$, respectively). *Trans*- ϵ -viniferin was only detected after 24 h pi and was not detected in water-treated Chasselas (Fig. 3c). For BABA-primed Chasselas, production started at 24 h pi, reaching a plateau at 48 h pi (28 $\mu\text{mol mg FW}^{-1}$). The highest concentration of ϵ -viniferin was found in water-treated Solaris at 48 h pi (97 $\mu\text{mol mg FW}^{-1}$) and then decreased to 56 $\mu\text{mol mg FW}^{-1}$ at 72 h. The production of ϵ -viniferin increased steadily in BABA-primed Solaris starting at 24 h until 72 h where it reached the same amount as in water-treated Solaris at 48 h pi. *Trans*- δ -viniferin accumulated transiently starting at 24 h pi and peaking at 72 h only for

Fig. 3 Quantitative analysis of the accumulation of several stilbenes in leaf samples of a susceptible and resistant cvs Chasselas and Solaris, respectively, over time after inoculation with *P. viticola*. Chasselas cuttings were treated with water (grey bars) or BABA (white bars) and Solaris cuttings were also treated with water (striped bars) or BABA (black bars) 2 days prior to inoculation. The analysed stilbenes were *trans*-piceide, *trans*-resveratrol, *trans*- ϵ -viniferin, *trans*- δ -viniferin and pterostilbene. Values presented are means \pm standard error of the mean. The experiment was repeated twice with similar results



water-treated Solaris and BABA-primed Chasselas and Solaris (Fig. 3d). The highest concentration was found in BABA-primed Solaris at $100 \mu\text{mol mg FW}^{-1}$, whereas that for BABA-primed Chasselas and water-treated Solaris the concentration of δ -viniferin was the same at $49 \mu\text{mol mg FW}^{-1}$ at 72 h pi. Pterostilbene was detected at relatively lower levels compared to the quantity of the other phytoalexins (Fig. 3e). It was only detected in BABA-primed cuttings, starting at 24 h pi for BABA-primed Chasselas and 12 h later for BABA-primed Solaris. Pterostilbene accumulated transiently for both BABA-primed cuttings peaking at 72 h pi with the same concentrations of $31 \mu\text{mol mg FW}^{-1}$ for both BABA-primed samples.

Response of grapevine genes involved in the phenylpropanoid biosynthesis pathway to *P. viticola* infection after treatment with and without BABA

The expression pattern of three genes involved in the phenylpropanoid biosynthesis pathway was analysed using quantitative real-time PCR: the first gene encodes phenylalanine ammonia lyase (*PAL*), the first enzyme of the pathway, the second gene encodes cinnamate-4-hydroxylase (*C4H*), the enzyme which catalyses the conversion of cinnamate into 4-hydroxy-cinnamate, a key reaction of the phenylpropanoid pathway, and the third gene encodes stilbene synthase (*STS*), the enzyme responsible for the synthesis of stilbenes such as piceides and resveratrol.

In infected, BABA-primed Solaris plants, the expression of *PAL* increased rapidly and transiently. There was no significant transcript accumulation of *PAL* in infected, BABA-primed Chasselas and in infected water-treated Chasselas and Solaris. The induction by BABA in infected Solaris peaked at 7 h with a maximum intensity 15-fold higher, than in both water-treated and BABA-primed infected Chasselas. Cinnamate-4-hydroxylase (*C4H*) was rapidly and transiently induced peaking at 7 h for both BABA-primed infected plants and for water-treated infected Chasselas. There was no significant change in expression of *C4H* in water-treated infected Solaris. In both BABA-primed infected plants *STS* was transiently induced, reaching maximum levels at 7 h (5-fold and 21-fold induction for Solaris and Chasselas, respectively). The level of expression of *STS* decreased for the first 24 h and increased again between 24 and

48 h pi. For both water-treated cultivars there was no significant accumulation of *STS* transcript levels for *STS*.

Discussion

The resistance inducer BABA has been shown to work mainly through priming of defence responses by sensitising the plants to respond faster and more adequately to the exposure to a given stress situation (Jakab et al. 2001; Conrath et al. 2002; Prime-A-Plant Group et al. 2006). Previous work in our group has shown that BABA induced resistance against *P. viticola* in both the susceptible Chasselas and the resistant Solaris cultivars of grapevine. This observed resistance in BABA-primed Chasselas plants as well as the basal resistance of Solaris depended to a large extent on the deposition of callose, which was positively correlated with BABA- and jasmonic acid-induced resistance (Hamiduzzaman et al. 2005). Here, we looked at the involvement of phytoalexins and investigated three genes involved in the phenylpropanoid biosynthesis pathway in BABA-IR in grapevine against downy mildew.

Phytoalexins have long been accepted as being important in the defence mechanisms of plants against phytopathogenic microorganisms. Previous work has demonstrated that two biochemical processes are indicative of downy mildew resistance in grapevines. One is the synthesis of callose in stomata (Gindro et al. 2003) and the second is the synthesis of resveratrol and its subsequent oxidation to ϵ - and δ -viniferins (Langcake 1981; Pezet et al. 2003, 2004a). The obvious priming for accumulation of different flavonoids in BABA-primed, infected grapevine plants compared to untreated water controls (Fig. 2d and f) observed at the microscopic level led us to further investigate the involvement of phytoalexins in BABA-IR at the biochemical and molecular level.

Not all stilbenes are equally toxic to *P. viticola* zoospores. Resveratrol is not a toxic compound as a consequence of its hydrophilic character (Dercks and Creasy 1989). According to Pezet et al. (2004a), piceide has never shown any toxic activity against *P. viticola* zoospores, even at concentrations greater than $1,000 \mu\text{M}$. In addition, resveratrol was found to be glycosylated to form piceide in susceptible cultivars (Gindro et al. 2003). In our experiment,

BABA-treatment led to an induction of *trans*-piceide accumulation in the susceptible Chasselas cultivar to levels higher than found in the resistant Solaris cultivar. BABA treatment did not seem to have any effect on piceide accumulation in Solaris (Fig. 3a). On the other hand BABA-treatment significantly induced the accumulation of *trans*-resveratrol in both susceptible and resistant cultivars. After 48 h pi the amount of *trans*-resveratrol in the BABA-primed susceptible Chasselas was equal to that found in the non-treated resistant Solaris and after 72 h pi the application of BABA led to an induction of *trans*-resveratrol in both cultivars to a comparable amount (Fig. 3b). In susceptible cultivars, resveratrol is synthesised in large amounts, but it is rapidly glycosylated into the non-toxic compound piceide, which could explain the high concentration of *trans*-piceide in BABA-primed Chasselas due to the high concentration of *trans*-resveratrol. This was not the case in non-treated Chasselas plants.

The oxidation products of resveratrol, the viniferins, are active against *P. viticola*; δ -viniferins is five times more toxic than ϵ -viniferin (Pezet et al. 2004b). Both ϵ - and δ -viniferin have been found in low concentrations in susceptible cultivars (Pezet et al. 2004a). We also found both viniferins in the susceptible Chasselas but only in BABA-primed plants at 48 h pi (Fig. 3c and d). Treatment of the resistant cv. Solaris with BABA did not seem to have a significant effect on the accumulation of ϵ - and δ -viniferins. It is important to note that an increase in resveratrol synthesis occurred at 48 h pi for BABA-primed Chasselas providing an important pool for the synthesis of viniferins that also occurred at 48 h pi.

Pterostilbene is as toxic for *P. viticola* as δ -viniferin, but it is usually absent or its concentration is too low to contribute to resistance mechanisms (Pezet et al. 2004a). We found no detectable amounts of pterostilbene in both water-treated Chasselas and Solaris; however, BABA-treatment induced a significant increase in pterostilbene in both infected cultivars even though the amounts were lower compared to the other stilbenes analysed. In BABA-primed Chasselas the level of pterostilbene at 72 h pi was comparable to that of BABA-primed Solaris (Fig. 3e).

Pezet et al. (2004a) concluded that there were two different types of reactions that could be observed between susceptible and resistant cultivars. In suscep-

tible cultivars, resveratrol is synthesised in large amounts after infection, but it is rapidly glycosylated into piceide, which is a non-toxic compound, whereas in resistant cultivars resveratrol is also synthesised in large amounts but it is rapidly oxidised into the more toxic viniferins. From our experiments BABA-treatment of the susceptible Chasselas cultivar made it respond more like resistant Solaris and more importantly BABA-treatment was able to prime the production of pterostilbene in both cultivars which, along with δ -viniferin, have been shown to be the most toxic stilbenes affecting zoospore mobility and disease development of *P. viticola* (Pezet et al. 2004b). BABA-treatment was also able to prime δ -viniferin accumulation in Chasselas to levels comparable to non-treated Solaris at 72 h pi but not as high as in BABA-primed Solaris (Fig. 3d).

The grapevine stilbenes are flavonoid-type phytoalexins and are formed via the phenylpropanoid pathway, and synthesis of these stilbenes only occurs if *PAL* genes and other genes encoding downstream enzymes are induced (Jeandet et al. 2002). In our experiments, only BABA-treatment of Solaris was able to prime the expression of *PAL* in the first 7 h pi. There was no induction in *PAL* expression in BABA-primed Chasselas or in water-treated Chasselas and Solaris (Fig. 4a). On the other hand BABA-treatment of Solaris also led to the priming of the expression of *C4H* in the first 7 h pi, whereas there was no significant induction in *C4H* expression in BABA-primed compared to water-treated Chasselas for the first 7 h pi. However, 24 h pi a significant decrease in *C4H* expression was observed in the water-treated Chasselas whereas in BABA-primed Chasselas the expression level of *C4H* was comparable to levels found in BABA-primed Solaris (Fig. 4b). In tobacco, Blount et al. (2000) showed that *PAL* activity and levels of phenylpropanoid compounds were reduced in leaves and stems of plants in which *C4H* activity had been genetically down-regulated. However, *C4H* activity was not reduced in plants in which *PAL* activity had been down-regulated by gene silencing (Blount et al. 2000). These authors hypothesised that flux into the phenylpropanoid pathway is controlled, at least in part, via feedback regulation of *PAL* sensed through the production of cinnamic acid. Furthermore, when reduced below a threshold of 20% to 25% of wild-type activity, *PAL* becomes a rate-limiting step for lignin biosynthesis in tobacco (Bate et al. 1994;

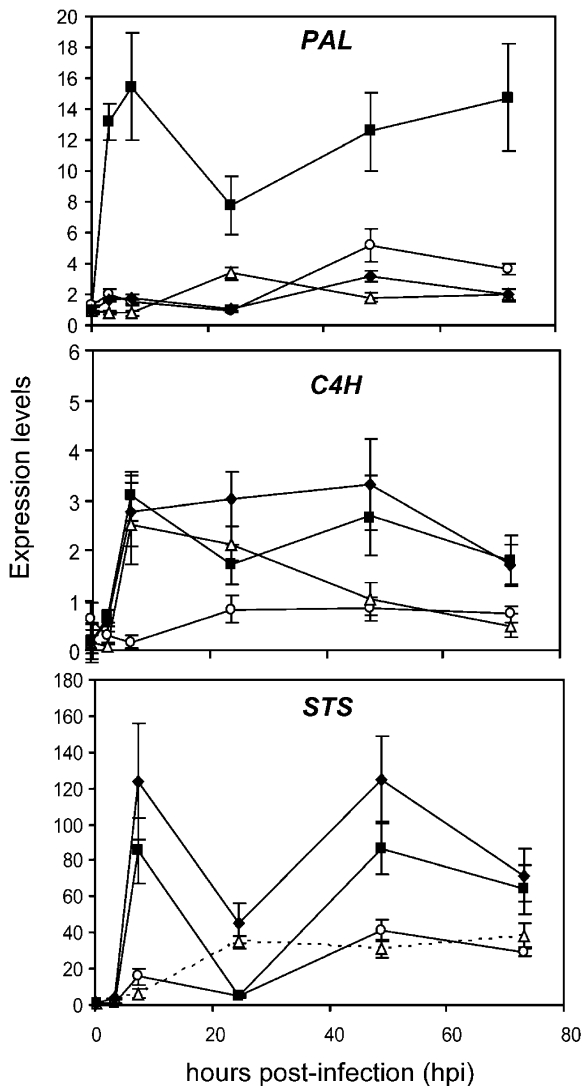


Fig. 4 Transcript accumulation of genes involved in the phenylpropanoid biosynthesis pathway. Leaf samples from the susceptible cv. Chasselas were treated with water (*open triangles*) or BABA (*closed diamonds*) 2 days prior to inoculation with *P. viticola*. The same water-treatment (*open circles*) or BABA-treatment (*closed squares*) was done for the resistant cv. Solaris. The three genes analysed by quantitative real-time PCR were: phenylalanine ammonia lyase (*PAL*), cinnamate-4-hydroxylase (*C4H*) and stilbene synthase (*STS*). Expression levels of the three genes were normalised relative to elongation factor 1- α . Values presented are means \pm standard error of the mean. The experiment was repeated twice with similar results

Howles et al. 1996). Reduced C4H activity is also correlated with reduced levels of lignin and phenolics (Sewalt et al. 1997). Even though BABA-treatment did not prime the expression of *PAL* in Chasselas, it primed the expression of *C4H*, which was maintained

at comparable levels to those in BABA-primed Solaris thereby allowing an increase in lignin biosynthesis for BABA-primed Solaris and Chasselas but not for the water-treated Solaris and Chasselas.

Stilbene synthase is a further key branch-point enzyme in the phenylpropanoid pathway leading to the production of resveratrol. The expression of *STS* is often induced in response to biotic and abiotic stresses. We found that at the transcriptional level, BABA-treatment of both resistant and susceptible cultivars induced an accumulation of *STS* expression in two peaks: one at 7 h pi and the other after 24 h pi (Fig. 4c). There was no priming of *STS* expression in either water-treated cultivars. Similar results were previously obtained with grapevine cell suspensions elicited with cell walls of *Botrytis cinerea* (Liswidowati et al. 1991) as well as with cell walls of *Phytophthora cambivora* (Wiese et al. 1994). The induction of *STS* expression found in the BABA-primed Chasselas and Solaris cultivars corresponded to an accumulation of resveratrol whereas a low *STS* gene expression in the water-treated cultivars corresponded to low levels of resveratrol (Figs. 3b and 4c).

Grapevine plants have various possibilities to defend themselves against an attack by *P. viticola*. As shown above, the phenylpropanoid pathway leading to the accumulation of phytoalexins as well as lignification has a major influence on the expression of resistance. However, the plants have also the possibility to mount other early defenses such as callose deposition at the stomatal entry points (Gindro et al. 2003; Hamiduzzaman et al. 2005). Since the addition of the PAL inhibitor AIP does not significantly compromise BABA-IR in Solaris (Hamiduzzaman et al. 2005), it seems likely that the contribution of callose deposition can compensate for the lack of phenylpropanoid pathway products. The exact contribution of each of these defense mechanisms to final resistance is, however, not yet clear.

Our results point to a prominent role for phytoalexins as a component of BABA-IR. Of special interest is the fact that BABA treatment is able to prime the plants to accumulate a specific phytoalexin, pterostilbene, that is otherwise not present in the plants. Interestingly, pterostilbene is extremely effective in interfering with zoospore mobility and general hyphal development and its specific priming might help to develop better methods of protection of grapes against downy mildew and possibly other diseases.

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