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Study of defense-related gene expression in grapevine leaves and berries infected with *Botrytis cinerea**

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Abstract

Defense responses of grapevine towards *Botrytis cinerea* were investigated. The expression of genes coding for proteins involved in defense were studied: (a) phenylalanine ammonia-lyase (PAL) and stilbene synthase (StSy), (b) an acidic chitinase (VCH3) and a basic chitinase (VCHIT1b), and (c) a polygalacturonase inhibitor protein (PGIP). Since no PGIP was known in grapevine, a complete cDNA sequence was first characterized by PCR and RACE-PCR amplifications. RNAs isolated from infected leaves and infected berries were analysed by semi-quantitative and real-time RT-PCRs. In infected leaves, the expression of PAL, StSy, PGIP and VCH3 genes occurred 6 hours post inoculation (hpi). Increase of VCHIT1b gene expression was delayed (24 hpi). Maximum levels of induction of these genes were observed at 48 hpi, except for the VCH3 gene (24 hpi). Activation of these defense responses was not sufficient to stop *B. cinerea* spread. In berries, no VCH3 gene expression was detected. Maximum levels of induction were observed in stage 3 (loss of berry colour and abundant production of conidia) for the PAL and PGIP genes, and in stage 4 (shrivelled berry) for the StSy and VCHIT1b genes.

Abbreviations: hpi – hours post inoculation; PAL – phenylalanine ammonia-lyase; PGIP – polygalacturonase inhibitor protein; StSy – stilbene synthase; RACE – rapid amplification of cDNA ends.

Introduction

Botrytis cinerea, the causal agent of grey mould, causes significant economic losses throughout the world as a destructive pathogen of many soft fruits, vegetables and flowers, particularly after harvest (Jarvis, 1980). In vineyards of Champagne, France, the disease affects both the yield of grape berries and the quality of wine. The fungus can also infect other parts of the grapevine plant such as the leaves and the woody stems.

Control against fungal diseases is currently achieved by application of fungicides. However, chemical control of *B. cinerea* is often difficult and incomplete (Gullino, 1992) especially in vineyards where resistant strains have developed (De Waard et al., 1993). A promising alternative strategy that could replace or be combined with fungicides is to stimulate the natural defense capabilities of plants by chemical inducers (Kessmann et al., 1994). In this perspective, it is important to unravel the molecular mechanisms of natural defense responses of grapevine to study the effect of these inducers and to point out the most effective defenses against *B. cinerea*.

Plants respond to pathogen invasion by inducing several defense reactions (Collinge and Slusarenko, 1987;

*Nucleotide and amino sequence data of the grapevine PGIP and of the two grapevine actins have been deposited in the GenBank database under the accession numbers AF305093, AF369524, AF369525, respectively.

Kombrink and Somssich, 1995). These include the synthesis of antimicrobial compounds (e.g. phytoalexins), polymers, such as callose and lignin, that form physical barriers, and pathogenesis-related (PR) proteins, which include hydrolytic enzymes capable of degrading components of pathogen cell walls. These defense responses have been studied during the interactions of *B. cinerea* with carrot, kiwi and tomato (reviewed by Elad, 1997). However, limited data are available concerning defense mechanisms induced during infection of grapevine. An increase in the content of the phytoalexin resveratrol was measured in *B. cinerea*-infected leaves and berries of grapevine (Langcake and Mc Carthy, 1979; Jeandet et al., 1995). Levels of chitinases and glucanases, or the corresponding activities increased in *B. cinerea*-infected grapevine leaves (Renault et al., 1996; Derckel et al., 1999). Accumulation of various mRNAs encoding PR proteins was also detected in leaves and/or berries in response to the causal agents of powdery and downy mildew (Busam et al., 1997a; Reuveni, 1998; Jacobs et al., 1999), and also during the development of grape berries (Tattersall et al., 1997; Kraeva et al., 1998; Salzman et al., 1998; Davies and Robinson, 2000).

In this study, the grapevine defenses were further characterized by cloning a polygalacturonase inhibitor protein (PGIP) gene. PGIPs are thought to interfere with fungal penetration by inhibiting the degradation of the plant cell wall by fungal endopolygalacturonases (PG). We also investigated the grapevine defense responses against *B. cinerea* by studying the expression levels of the PGIP gene and the genes coding for proteins involved in two other different types of plant responses: PAL and StSy (Sparvoli et al., 1994) providing lignins and resveratrol, respectively; and two chitinases (Busam et al., 1997a), a basic (VCHIT1b) and an acidic one (VCH3), which may hydrolyse chitin in *B. cinerea* cell wall. Leaves were infected under laboratory conditions and naturally infected berries were collected from vineyards. Accumulation of gene transcripts was studied with two different approaches: semi-quantitative and real-time RT-PCRs.

Materials and methods

Fungal and plant cultures

B. cinerea isolate T4 was a generous gift of Dr. Y. Brygoo (INRA, France). It was maintained as described by Derckel et al. (1999). Grapevine plants (*Vitis vinifera* cv. Chardonnay 7535) were micropropagated *in vitro* at 25 °C with a 16/8 h photoperiod (Derckel et al., 1999). Eight-week-old plantlets were used.

Infection procedures

Leaves. *B. cinerea* isolate T4 was grown in liquid culture for 10 days under shaking at 160 rpm at 21 °C with a 16/8 h photoperiod (Derckel et al., 1999). The culture was ground for 30 s in a blender and used to inoculate a new medium (5 ml of ground mycelium per 100 ml culture), which was incubated for a further 3 days. About 2 g of fresh young mycelium per 100 ml culture was obtained under these conditions. The culture was then ground for 30 s, glycerol was added to a final concentration of 10% (V/V) and the ground mixture was stored at −20 °C until use. Leaves detached from *in vitro*-cultivated grapevine plants were placed lower face up on wet paper in Petri dishes. The tip of 200 µl pipettors was gently applied to the surface of the leaves to induce small wounds without punching out tissue. About 16–17 wounds per 100 mg of leaf tissue were produced. A 3 µl droplet of ground mycelium was spotted onto each wound and leaves were incubated at 21 °C with a 16/8 h photoperiod, before harvesting at different times after treatment. As controls, leaves were mock-inoculated in the same way with sterile non-inoculated medium for 24 h before harvesting and non-treated leaves were directly harvested. Harvested tissues were frozen in liquid nitrogen and stored at −80 °C. Each assay was conducted two times on three separate plants.

Berries. Mature clusters (*V. vinifera* cv. Pinot Noir clone 389) of berries partially infected by *B. cinerea* were collected in the vineyard and sorted according to four different stages of infection: stage 1 was healthy berry, stage 2 was when the first infection spot was visible on the berry, stage 3 was when loss of berry colour and abundant production of conidia were visible, stage 4 was when the berries had shrivelled. Harvested berries were frozen in liquid nitrogen and stored at −80 °C until use.

Nucleic acids extraction

DNA was extracted from plant leaves and fungal mycelium (Steenkamp et al., 1994). Total RNA was extracted from berries (Davies and Robinson, 1996).

Messenger RNA was extracted from plant leaves and *B. cinerea* mycelium with magnetic beads (Dynal, France) according to the manufacturer's instructions with changes in extraction buffer (Tris-HCl 600 mM pH 7.5, LiCl 500 mM, EDTA 10 mM pH 8, LiDS 1.5%, sodium deoxycholate 1.5%, NP-40 1.5%, DTT 10 mM β-mercaptoethanol 1%).

PGIP cloning

Four degenerate primers (PGIP-F1 5′-KITGYAAYCC IVAIGAYAARMA-3′, -F2 5′-RYIYTIYTICARATH AARAARG-3′, -R1 5′-TCIARYWTRTTICKIYWIA RRTC-3′ and -R2 5′-ATYTSICCRCAIARIYKRTT RWA-3′) were designed, based on conserved amino acid sequences of PGIPs from bean (Toubart et al., 1992), pear (Stotz et al., 1993), tomato (Stotz et al., 1994) and kiwifruit (Simpson et al., 1995). A first PCR was performed using 100 ng of genomic DNA extracted from Chardonnay cultivar leaves with the outer primers PGIP-F1/-R2. One microlitre of this primary PCR product was reamplified using the inner primers PGIP-F2/-R1. The resulting 558 bp product was cloned into pGEM-T easy vector (Promega, France) and three clones were sequenced. Based on the sequence of this product, gene-specific primers (PGIP-R3 5′-GACATTGGGGTCGAATCCTC-3′, PGIP-5′ 5′-TCGGGAATCTGGCCGGATAGCTGGC CGG-3′, PGIP-3′ 5′-CGCCGGCTCTACCCCAGGT CTACACCTC-3′) were designed and used in RACE-PCR to amplify the 5′ and 3′ ends of the PGIP transcript.

For the 3′ end cloning, cDNA was amplified from RNA with the SMART PCR cDNA synthesis kit (Clontech, France) according to manufacturer's instructions. Twenty five cycles of PCR (one cycle was 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) was performed using 2 µl of SMART cDNA with PGIP-3′ and an oligo-dT_{21} as primers. The resulting 556 bp product was cloned into pGEM-T easy vector (Promega, France) and four clones were sequenced.

5′-RACE was performed from RNA using the SMART RACE cDNA amplification kit (Clontech, France). A first amplification was performed with PGIP-R3 and Universal Primer Mix supplied with the kit for 25 cycles (one cycle was 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C). One microlitre of this primary PCR was reamplified using the Nested Universal Primer supplied with the kit and PGIP-5′ for 25 cycles (one cycle was 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C). The resulting 379 bp product was cloned into pGEM-T easy vector (Promega, France) and four clones were sequenced.

Partial grapevine cDNA actin cloning

Degenerate primers (dVACT-F 5′-GATATGGAGA ARATMTGGCATCAYAC-3′, dVACT-R 5′-GTTTCR TGAATWCCWGCWGCTTCC-3′) specific to actin genes cloned from tobacco and *Arabidopsis thaliana* plants (Moniz de Sa and Drouin, 1996; An et al., 1996) were designed. A 30 cycle PCR (one cycle was 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C) was performed from 2 µl of SMART cDNA. The resulting 589 bp product was cloned into pGEM-T easy vector (Promega, France) and three clones were sequenced. The sequence of this product was compared to *B. cinerea* actin gene sequence (Benito et al., 1998) and non-degenerate primers specific for the grapevine actin gene were designed (VACT-F 5′-ATGTGCCTGCCATGTATGTTGCC-3′, VACT-R 5′-AGCTGCTCTTTGCAGTTTCCAGC-3′).

Sequence analysis

Sequencing of both strands was performed with the dideoxynucleotide chain termination reactions by P. Hammann (IBMP, Strasbourg, France). Protein sequence comparisons were done using the BLAST program (Altschul et al., 1997) on the network (National Center for Biotechnology Information, Bethesda, MD). Alignment of predicted amino acid sequences of grapevine PGIP with the homologous proteins was achieved using the ClustalW 1.7 programme.

Determination of mRNA level

Semi-quantitative RT-PCR analysis. RNA (160 ng) from the different leaf samples were reverse transcribed in the presence of oligo$(dT)_{21}$ primer and H_2O was added to a final volume of 100 µl. Primers VACT-F and VACT-R specific for the constitutively expressed grapevine actin gene were used in a PCR on non-reverse transcribed RNA samples to test for DNA contamination of the RNA preparation, and in RT-PCR to normalyze the different samples for differences in the amounts of plant RNA. The following PCR conditions were used: 94 °C, 60 °C and 72 °C, each for 1 min, for 25 cycles in a final volume of 50 µl. For each pair of specific primers, PCR conditions were determined to

perform the amplification within the exponential phase as follows: a first PCR was performed on 1 µl of RT from each sample to reveal the one for which the amplified product was the most abundant. A second PCR was performed on dilutions of the RT of this sample to determine an adequate quantity of RT to achieve amplification within the exponential phase. This quantity was applied to all samples to perform a third PCR. 10 µl of product from this third PCR was resolved on agarose gel stained with ethidium bromide to visualize the gene expression level.

Real-time RT-PCR analysis. RNAs from leaves and primers used were the same as for semi-quantitative RT-PCR analysis. RT of RNA from berries was performed with 0.5–1 µg of total RNA. RT-PCR reactions were carried out in 96-well plates (25 µl per well) in a reaction buffer containing 1× SYBR Green I mix (PE Biosystems; including Taq polymerase, dNTPs, SYBR Green dye), 300 nM primers (forward and reverse) and 3.2–1600 pg of reverse transcribed RNA depending on experiments. PCR conditions were 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension) for 40 cycles on a GeneAmp 5700 sequence Detection System (PE Biosystems). PCR efficiency for each target mRNA was obtained via the slope of the standard curve. Three standard curves were determined: one from infected leaves, one from berries at stage 1 and one from berries at stage 4. Linearity was obtained from a range of 3.2–160 pg of reverse transcribed RNA obtained from infected leaves harvested 48 hpi and from a range of 32–1600 pg of reverse transcribed RNA obtained from berries at stages 1 and 4. The absence of primer-dimer formation, which could interfere with specific amplification, was checked in no template controls. Each time point was determined as an average from data obtained from triplicate. Relative gene expression was obtained with the formula fold induction $= 2^{-[\Delta\Delta C_T]}$, where $\Delta\Delta C_T = [C_T\,\mathrm{GI(unknown\ sample)} - C_T\,\mathrm{VACT\ (unknown\ sample)}] - [C_T\,\mathrm{GI(calibrator\ sample)} - C_T\,\mathrm{VACT(calibrator\ sample)}]$. The C_T (threshold cycle) value represents the PCR cycle at which the copy number passes the fixed threshold and can be first detected. GI is the gene of interest and VACT is the grapevine actin gene used as internal control. The calibrator sample is the sample chosen to represent 1× expression of the gene of interest (e.g. non-treated leaves). For more details see the user's manual and Winer et al. (1999).

Results

PGIP cDNA cloning

A combination of four degenerate primers used in two successive PCR amplifications yielded a 558 bp product, which corresponded to the central part of a grapevine PGIP gene. Based on the sequence of this product, gene-specific primers were used in RACE-PCR to amplify the 5′ and 3′ ends of the PGIP transcript. The complete sequence of the cDNA encoding grapevine PGIP, derived from the three overlapping partial clones, was 1158 bp long with an open reading frame encoding a predicted polypeptide of 333 amino acids (Figure 1). A comparison of grapevine PGIP with other available PGIP sequences in databases revealed that this clone shared 70% identity at the amino acid sequence level with PGIPs from kiwifruit (Simpson et al., 1995), pear (Stotz et al., 1993) and apple (Yao et al., 1999), and 71%, 69%, 62% and 46% identity with PGIPs from apricot (unpublished, accession number AF020785), lemon (unpublished, accession number AB016206), tomato (Stotz et al., 1994) and bean (Toubart et al., 1992), respectively (Figure 2).

Grapevine actin cDNA cloning and design of primers that discriminated between grapevine and B. cinerea *actin genes in RT-PCR amplification*

Degenerate primers specific for tobacco and *A. thaliana* actin genes were designed to amplify a partial actin cDNA from grapevine. The 590 bp fragment obtained was cloned and three different clones were sequenced. Two clones were identical, one exhibited 11 different nucleotides, five of which were localized in the degenerate primers (data not shown). At the amino acid level one residue was different between the two different clones. Protein coded by the two identical clones shared 98% and 97% identity with actin from *A. thaliana* and tobacco, respectively (data not shown). Comparison of this grapevine actin gene sequence with the *B. cinerea* actin gene sequence (Benito et al., 1998) using the BLASTN programme revealed 75% identity at the nucleotidic level (data not shown). Based on this comparison, two specific primers VACT-F and VACT-R were designed in a region where very low homology exists between grapevine and fungus actin genes (primers of 23 nucleotides with 10 and 13 nucleotides difference, respectively) to amplify

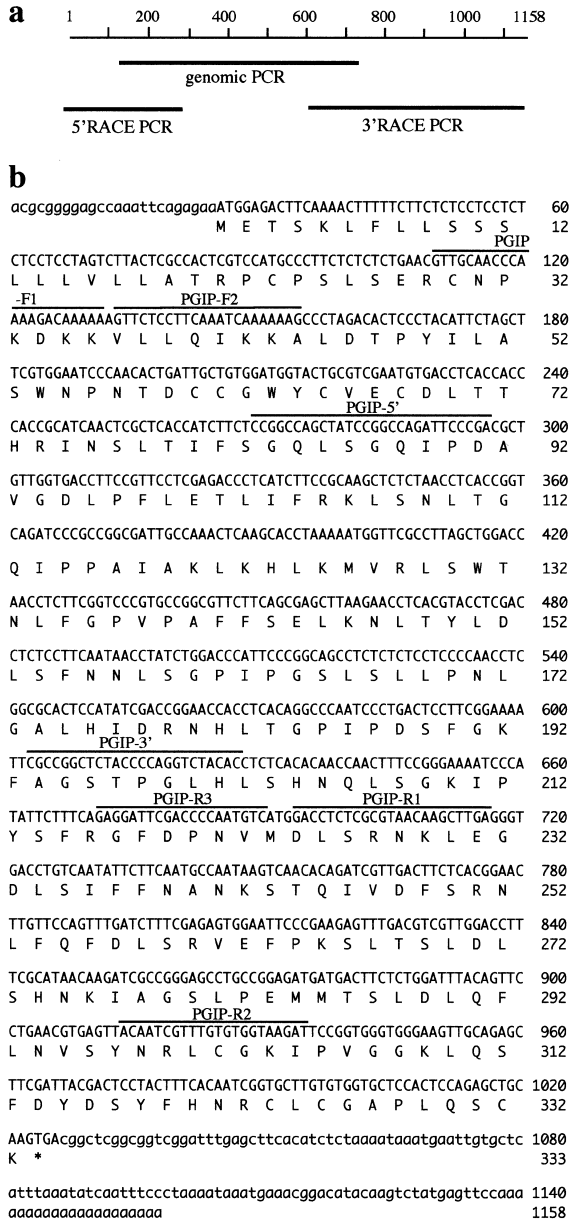

Figure 1. Cloning strategy and sequence of PGIP cDNA. (a) The three overlapping PCR products and their location. Nucleic acid number is based on the deduced sequence. (b) Nucleotide and deduced amino acid sequences of the grapevine PGIP gene. Positions of the different primers are indicated.

plant actin cDNA in a RT-PCR experiment. In non-treated plants a fragment corresponding to actin from grapevine was amplified (Figure 3a, VACT, lane NT), but in *B. cinerea* grown in sterile liquid culture no transcript was detected (Figure 3a, VACT, lane Bc).

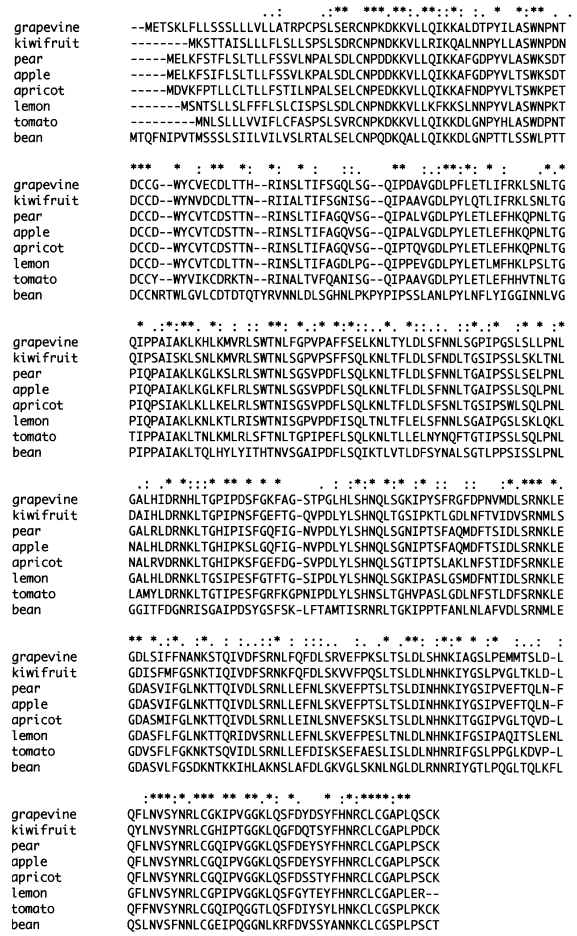

Figure 2. Comparison of predicted amino acid sequence of grapevine PGIP with the PGIP proteins from kiwifruit (Simpson et al., 1995), pear (Stotz et al., 1993), apple (Yao et al., 1999), apricot (accession number AF020785), lemon (accession number AB016206), tomato (Stotz et al., 1994) and bean (Toubart et al., 1992). '*' indicates positions that have a single, fully conserved residue; ':' indicates that one residue of a strong group is fully conserved, and '.' indicates that one residue of a weaker group is fully conserved (see ClustalW 1.7 for details).

In the latter situation, a transcript was detected when RT-PCR was performed with primers specific from the fungal actin gene (data not shown). These results indicated that the VACT primers discriminated between fungal and plant actin genes.

Expression of grapevine defense-related genes during the infection process in leaves

Expression of grapevine defense-related genes coding for PAL, StSy, an acidic chitinase, VCH3, a basic

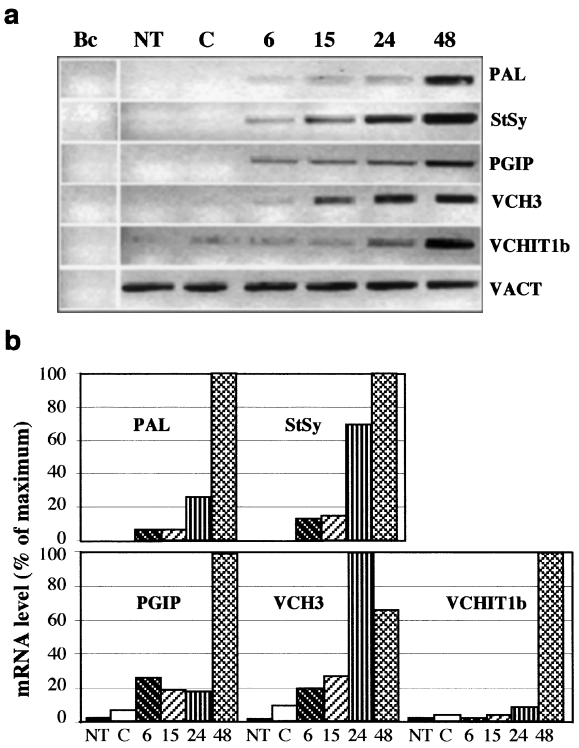

Figure 3. Time course change of mRNA level of defense-related genes in leaves during the infection progress. Analyses were performed by (a) semi-quantitative and (b) real-time RT-PCRs. Both approaches were performed on the same cDNA samples obtained by RT of RNA extracted from *B. cinerea* grown *in vitro* (Bc), from non-treated plants (NT), from mock-inoculated leaves harvested 24 h after treatment (C) and from *B. cinerea*-infected leaves harvested 6, 15, 24 and 48 hpi (6, 15, 24 and 48 respectively). Defense-related genes studied were phenylalanine ammonia-lyase (PAL), stilbene synthase (StSy), polygalacturonase inhibitor protein (PGIP), acidic chitinase (VCH3), and basic chitinase (VCHIT1b). (a) Grapevine actin gene was used as internal control to normalize the different samples with the same amount of plant RNA (VACT). The quantity of template was adapted for each pair of primers to perform amplification within the exponential phase (value referred ng RNA before RT in non-treated plants): 1.2 ng for VACT, 0.2 ng for PAL, 0.1 ng for StSy, 1.6 ng for PGIP and VCH3, and 0.8 ng for VCHIT1b. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. Results presented are typical data obtained from two independent experiments. (b) For each pair of primers, RT-PCR was performed on a quantity of template corresponding to 16 pg of RNA. Relative fold induction was calculated using the $\Delta\Delta C_T$ method from triplicate data, with grapevine actin gene as internal control and non-treated leaves as 1× expression level (see Materials and methods). Subsequently, for each gene, the sample with maximum fold induction was referred to as 100% expression level. NT and C for PAL and StSy: Expression was too low to be indicated as a bar.

chitinase, VCHIT1b and PGIP in response to *B. cinerea* infection was analysed on leaves. Use of detached leaves from *in vitro* cultivated grapevine plantlets and ground mycelium instead of conidia resulted in efficient and synchronous infection. All lesions on a single leaf grew at equal rates and no significant difference in lesion growth was observed between leaves. This allowed the analysis of defense gene expression in a synchronous time course experiment. Induction of defense-related genes was studied by semi-quantitative RT-PCR and real-time RT-PCR approaches (Figures 3a and b, respectively). As the infection progressed, the fungal biomass within the host tissue increased. Consequently, the proportion of fungal RNA present in the RNA extracted from infected plant tissue increased with time while the proportion of plant RNA decreased. This decrease could introduce a bias in the quantification of RNA of defense-related genes during the infection process. Grapevine actin gene was selected as an internal control to normalize the different samples for differences in the amount of plant RNA. We considered that both approaches gave similar results even if some differences could be noticed in induction levels for some timepoints (Figure 3). No amplification was obtained when RT-PCR was performed from RNA extracted from *B. cinerea* grown in liquid culture (Figure 3a, lanes Bc) or from genomic DNA of the fungus (data not shown) indicating that all primers were specific for grapevine. With the semi-quantitative RT-PCR approach the expression of defense-related genes was undetectable in non-treated plants except for VCHIT1b where a weak basal constitutive expression was detected (Figure 3a, lanes NT). It should be noted that in the first PCR (see Materials and methods) a weak basal level of expression was detected for all genes (data not shown). However, the low template quantity used in the third PCR to perform the amplification in the exponential phase for all samples did not allow visualization of an amplified fragment in non-treated plants. On the other hand, with the real-time RT-PCR approach, a weak basal constitutive expression was detected in non-treated plants for all genes. This level of expression was chosen to represent 1× expression of the different genes. Then all expression levels were normalized to 100%, the maximum fold of induction measured for each gene (Figure 3b). Both RT-PCR approaches revealed a slight induction for some genes in the mock-inoculated plants compared to non-treated plants (Figure 3, lanes C). These results indicated that the *in vitro* experimental procedure of

infection possibly caused a weak wounding stress to leaves that only slightly modulated gene transcript levels. In *B. cinerea*-infected leaves, all defense-related genes studied were up-regulated. Expression levels of PAL, StSy, PGIP and VCH3 genes rose by 6 hpi (Figures 3a and b, lanes 6). The increase of VCHIT1b gene expression was delayed (24 hpi). Maximum levels of induction of the genes were observed at 48 hpi except for VCH3 gene (24 hpi).

Expression of grapevine defense-related genes in berries at different stages of infection

The study of defense gene expression in berries was performed with naturally field-infected berries sorted according to four different stages of infection. Experiments were performed with the same real-time RT-PCR approach as done with infected leaves (Figure 4). No VCH3 gene expression was measured in healthy berries or in any stage of infection, which indicated a clear difference in the expression of the gene between leaves and berries. There was no expression of the VCHIT1b gene in healthy berries, but it appeared at stage 2. So this level of expression was chosen to represent 1× expression of VCHIT1b. PAL, StSy and PGIP gene expression were detected in healthy berries and this was assigned to be the 1× expression level. Expression of the four genes was up-regulated in the infected material at all stages. Induction was maximum in stage 3 for PAL and PGIP genes and in stage 4 for StSy and VCHIT1b genes (Figure 4).

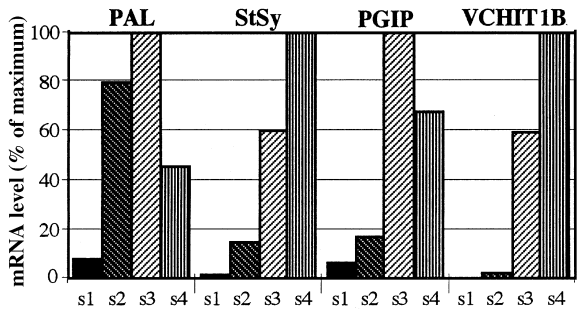

Figure 4. Analysis of defense-related gene expression in berries at different stages of infection by real-time RT-PCR. RT-PCR was performed on RNA extracted from healthy berries (stage 1), from berries with a first visible infection spot (stage 2), from berries with loss of colour and visible abundant production of conidia (stage 3), and from shrivelled berries (stage 4). Conditions were the same as in Figure 3b, except that the quantity of template corresponded to 160 pg of RNA. The 1× expression level was stage 1, except for VCHIT1b gene (stage 2).

Discussion

PAL, StSy, VCH3 and VCHIT1b genes had been previously characterized in grapevine (Sparvoli et al., 1994; Busam et al., 1997a), but their expression has not been studied in grapevine leaves and berries infected with *B. cinerea*. No PGIP gene had been characterized in grapevine. The results of this study demonstrated that grapevine responded to *B. cinerea* infection with up-regulation of several defense-related genes. Gene induction took place in infected leaves as well as in infected berries. Surprisingly, in this latter case, even in a shrivelled state, berries were still able to induce several defense-related genes. However, corresponding protein accumulation was not analysed.

The accumulation of the transcripts corresponding to two cDNAs encoding chitinases was demonstrated. The defense profile was not the same between infected leaves and berries as VCH3 mRNA was only detected in leaves. The presence of different chitinase isoforms within leaves and berries was previously reported in healthy tissues (Derckel et al., 1996; Robinson et al., 1997) or following infection by powdery mildew (Jacobs et al., 1999). In infected leaves, the VCH3 gene was induced earlier than the VCHIT1b gene. The same observation was previously noticed in grapevine cells treated with an elicitor (Busam et al., 1997a), and a difference in kinetics between VCH3 and another basic chitinase (VvChi1b) was also reported in leaves in response to powdery mildew infection (Jacobs et al., 1999). Induction of VCH3 gene was measured as early as 6 hpi, as found for PAL and StSy genes. PR proteins are usually induced later than phytoalexin biosynthetic enzymes, but in grapevine cells treated with a yeast extract, induction of VCH3 gene occurred as early as 2 h post treatment as for StSy and O-methyltransferase genes (Busam et al., 1997a,b). We did not study whether similar kinetics of induction occured before 6 hpi in *B. cinerea*-infected leaves. These results suggest that in grapevine, VCH3 gene induction can occur at the same early timepoints as for phytoalexin biosynthetic enzymes genes regardless of the inducer, elicitor or pathogen. Early induction of a chitinase gene, in the same kinetics than a PAL gene, was also previously found in sunflower hypocotyls infected with downy mildew (Mazeyrat et al., 1999). Low levels of VCHIT1b transcripts were shown to be constitutively expressed in grapevine leaves (Busam et al., 1997a), and no induction in response to downy mildew infection was found (Busam et al., 1997a). We also measured a weak constitutive expression of the VCHIT1b

gene, but in addition, the gene was induced in response to *B. cinerea* infection. These results suggest that grapevine expresses a different set of defense genes in response to different pathogens.

We have isolated and characterized a PGIP gene, which was induced in response to *B. cinerea* infection. Gene induction was measured as early as 6 hpi. This result suggests that, like PAL, StSy and VCH3, the PGIP gene is part of an early defense response in grapevine. In contrast, the expression of VCHIT1b is delayed. We did not find any other report that compared PGIP expression with other defense genes. PGIPs inhibit the activity of PGs secreted by phytopathogenic fungi and increase the stability of elicitor-active oligogalacturonides *in vitro* (Cervone et al., 1989). Therefore, PGIPs could contribute to the defense mechanisms of plants against pathogens. A gene coding for a PG has been shown to be required for full virulence of *B. cinerea* (ten Have et al., 1998) and heterologous expression of a pear PGIP in tomato reduced the growth of *B. cinerea* (Powell et al., 2000). In a crude protein extract prepared from green berries, we measured a thermolabile PGIP activity against *B. cinerea* PG activity in *in vitro* assays (F. Baillieul, unpublished results). Taken together, these results suggest that PGIP could be an important aspect of the induced resistance against *B. cinerea* in grapevine.

In the present study, the defense responses were not sufficient to stop *B. cinerea* from spreading and after 2–3 days leaves were completely infected and necrotic. In a previous study, the strain T4 inoculated via spores on intact leaves did not generate spreading lesions and was considered a non-aggressive strain on grapevine (Derckel et al., 1999). No gene-for-gene resistance has been identified in plant–*B. cinerea* interactions and environmental factors can affect the balance between the plant's defenses and the pathogen's ability to overcome them (Elad and Evensen, 1995). So the greater aggressiveness observed in the present study might be assigned to the procedure of inoculation which favours the fungus compared to the plant. Therefore chemical treatments that enhance defense capabilities of grapevine could allow the plant to escape disease from aggressive *B. cinerea* isolates or to slow down the spread of the disease. Production of some β-1,3-glucanases in grapevine leaves treated with SA has been reported (Renault et al., 1996). Grapevine cells treated with SA and INA exhibited an increase in the expression of VCH3, VCHIT1b and StSy genes while treatment with BTH induced only the VCH3 gene (Busam et al., 1997a,b). These results suggested that grapevine was capable of expressing defense responses in response to chemical inducers but that gene expression was different according to the chemical inducer. No subsequent protection against pathogen attack was studied. In the vineyard, grapevine plants treated with an association of seaweed extract (i.e. mix of crude elicitors) and aluminium chloride were more resistant to *B. cinerea* (Jeandet et al., 1996). This was correlated to resveratrol induction. Expression of other defense responses was not studied. In the present study, a new grapevine defense marker, PGIP was characterized, and an up-regulation of various defense genes in *B. cinerea*-infected leaves and berries was demonstrated. These results validate our model to be useful to further characterize grapevine defense responses at the molecular level. A differential screening technique (suppression subtractive hybridization or cDNA-AFLP) should be considered. Grapevine defenses genes and products will provide markers for a better understanding of the action of the plant resistance inducers in grapevine. It will allow a correlation to be established between the defense responses expressed after treatment by inducers and the level of resistance observed against *B. cinerea* infection in treated plants.

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